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H. P. TALBOT, Ph.D., Sc.D., CONSULTING EDITOR

**PROTEINS AND THE THEORY OF
COLLOIDAL BEHAVIOR**

INTERNATIONAL CHEMICAL SERIES

(H. P. TALBOT, PH.D., SC.D., CONSULTING EDITOR)

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PROTEINS AND THE THEORY OF COLLOIDAL BEHAVIOR

BY

JACQUES LOEB

Member of the Rockefeller Institute for Medical Research

SECOND EDITION
SECOND IMPRESSION

McGRAW-HILL BOOK COMPANY, INC.
NEW YORK: 370 SEVENTH AVENUE
LONDON: 6 & 8 BOUVERIE ST., E. C. 4
1924

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MCGRAW-HILL BOOK COMPANY, INC.

PRINTED IN THE UNITED STATES OF AMERICA

THE MAPLE PRESS COMPANY, YORK, PA.

PREFACE TO THE SECOND EDITION

Following the appearance of the first edition of this book the author carried on numerous experiments which contributed important corroborative evidence of the theory for colloidal behavior developed by him. The results of these recent experiments were incorporated in the French and German editions of the book. The second edition in English was prepared in accordance with the foreign editions by the author before his death. The arrangement of material has been made by Miss N. Kobelt who has also read the proof with the assistance of Dr. Anne Leonard Loeb.

ROBERT F. LOEB.

NEW YORK

PREFACE TO THE FIRST EDITION

Colloid chemistry has been developed on the assumption that the ultimate unit in colloidal solutions is not the isolated molecule or ion but an aggregate of molecules or ions, the so-called micella of Naegeli. Since it seemed improbable that such aggregates could combine in stoichiometrical proportions with acids, alkalies, or salts, the conclusion was drawn that electrolytes were adsorbed on the surface of colloidal particles according to a purely empirical formula, Freundlich's adsorption formula.

The writer's investigations have led to the result that this last conclusion is due to a methodical error, as far as the proteins are concerned; namely, the failure to measure the hydrogen ion concentration of the protein solutions, which happens to be one of the main variables. When the hydrogen ion concentrations are duly measured and considered, it is found that proteins combine with acids and alkalies according to the stoichiometrical laws of classical chemistry and that the chemistry of proteins does not differ from the chemistry of crystalloids.

As long as chemists continue to believe in the existence of a special colloid chemistry differing from the chemistry of crystalloids, it will remain impossible to explain the physical behavior of colloids in general and of proteins in particular. This state of affairs is reflected in the concluding remarks of Burton's interesting book on "The Physical Properties of Colloidal Solutions," published in 1916:

We may very well conclude with the words used by the pioneer worker Zsigmondy, in closing his first account of the early work on colloidal solutions:

"From the foregoing outline no general theory of colloids can be given, for the study of colloids has become a great and extensive science, in the development of which many must assist; only when the voluminous material supplied by much physico-chemical research has been properly systematized will the theory of colloidal solutions be raised from mere consideration of the similarities in special cases to the standing of an exact science."

Professor F. G. Donnan, of the University of London, announced in 1910 an ingenious theory of equilibria which are established when two solutions of electrolytes are separated by a membrane which is permeable to all except one ion. This theory was successfully applied by Procter and Wilson to the explanation of the influence of electrolytes on the swelling of gelatin. It will be shown in this volume that Donnan's theory of membrane equilibria furnishes a quantitative and mathematical explanation not only of swelling but of the colloidal behavior of protein solutions in general; namely, electrical charges, osmotic pressure, viscosity, and stability of suspensions. Such an application of Donnan's theory would have been impossible without the stoichiometrical proof that proteins form true ionizable salts with acids and alkalis. What was at first believed to be a new type of chemistry, namely colloid chemistry, with laws different from those of general chemistry, now seems to have been only an unrecognized equilibrium condition of classical chemistry; at least as far as the proteins are concerned. This does not detract from the importance of colloidal behavior for physiological and technical problems, but it completely changes the theoretical treatment of the subject.

Any rival theory which is intended to replace the Donnan theory must be able to accomplish at least as much as the Donnan theory, *i.e.*, it must give a quantitative, mathematical, and rationalistic explanation of the curves expressing the influence of hydrogen ion concentration, valency of ions, and concentration of electrolytes on colloidal behavior; and it must explain these curves not for one property alone but for all the properties, electrical charges, osmotic pressure, swelling, viscosity, and stability of solution, since all these properties are affected by electrolytes in a similar way.

The contents of the book are divided into two parts, one furnishing the proof of the stoichiometrical character of the reactions of proteins, the second developing a mathematical and quantitative theory of colloidal behavior on the basis of Donnan's theory of membrane equilibria.

The theory of colloidal behavior, as outlined in this book, can only be considered as a first approximation. Finer methods of experimentation will have to be introduced, many minor dis-

crepancies will have to be accounted for, and many additions made. It was, however, thought advisable to publish the book for the reason that the experimental facts are accumulating so rapidly that it is difficult for anyone to gather the leading ideas unless they are presented more systematically and with less detail than in the original publications. It was also thought advisable to avoid in this volume a discussion of the possible applications of the new theory to physiological and technical problems.

The writer wishes to express his appreciation to his technical assistants, Mr. M. Kunitz, and Mr. N. Wuest, for the skill and care shown in the measurements required for the experimental part of the work.

The writer's thanks are also due to Dr. John H. Northrop, Dr. D. I. Hitchcock, and Dr. Anne Leonard Loeb, who have read part or all of the manuscript and offered valuable suggestions; and to Dr. J. A. Wilson, who kindly read and revised the first part of the chapter on swelling and suggested to the writer the mathematical proof on page 143 of the first edition.

The writer is indebted to Miss N. Kobelt for the reading of the proof and for the index.

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NEW YORK, N. Y.
March, 1922

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PART I
CRYSTALLOIDAL AND COLLOIDAL
BEHAVIOR OF PROTEINS

PROTEINS AND THE THEORY OF COLLOIDAL BEHAVIOR

CHAPTER I

HISTORICAL INTRODUCTION

1. THE ALLEGED DIFFERENCE BETWEEN THE CHEMISTRY OF COLLOIDS AND OF CRYSTALLOIDS

The distinction between crystalloids and colloids was proposed by Graham in 1861, the crystalloids being characterized by a tendency to form crystals when separating from an aqueous solution, and the colloids by a tendency to separate out in the form of "gelatinous" (or amorphous) masses. Graham found that these two groups of substances differ also in two other respects, first, in their "diffusive mobility," and second, in a peculiar "physical aggregation." The crystalloids diffuse readily through different kinds of membranes (*e.g.*, pig's bladder, parchment), through which colloids can diffuse not at all or only very slowly. The second peculiarity is the tendency of the colloids to form aggregates when in solution, while this property is lacking or less pronounced in crystalloids. A brief quotation from a paper by Graham will illustrate these definitions:

Among the latter [*i.e.*, the substances with low order of diffusibility] are hydrated silicic acid, hydrated alumina, and other metallic peroxides of the aluminous class, when they exist in the soluble form; and starch, dextrin, and the gums, caramel, tannin, albumen, gelatin, vegetable, and animal extractive matters. Low diffusibility is not the only property which the bodies last enumerated possess in common. They are distinguished by the gelatinous character of their hydrates. Although often largely soluble in water, they are held in solution by a most feeble

force. They appear singularly inert in the capacity of acids and bases, and in all the ordinary chemical relations.¹ But, on the other hand, their peculiar physical aggregation, with the chemical indifference referred to, appears to be required in substances that can intervene in the organic processes of life. The plastic elements of the animal body are found in this class. As gelatin appears to be its type, it is proposed to designate substances of the class as *colloids*, and to speak of their peculiar form of aggregation as the *colloidal condition of matter*. Opposed to the colloidal is the crystalline condition. Substances affecting the latter form will be classed as *crystalloids*. The distinction is no doubt one of intimate molecular constitution.²

It is therefore obvious that there are, according to Graham, at least two essential differences between colloids and crystalloids, the difference in diffusion through membranes, and the difference in the tendency to form aggregates in solutions. We shall see in this volume that the chief characteristics of colloidal behavior can be explained mathematically from the difference in diffusibility between colloids and crystalloids, while the tendency of certain protein molecules to form aggregates plays only a minor rôle.

In modern colloid chemistry it has, however, become customary to consider the tendency of colloids to form aggregates as the fundamental property, for the reason that the precipitation of colloids was the chief topic of research and discussion in colloid chemistry, and precipitation is, of course, due to the formation of aggregates. The colloidal state is defined by colloid chemists as that state of matter in which the ultimate units in solutions are no longer isolated molecules or ions, but aggregates of molecules for which Naegeli had introduced the term *micella* (small crumb). Thus, Zsigmondy states:

. . . that the essential and characteristic constituents of colloidal solutions are very small ultramicroscopic particles the dimensions of which lie between molecular and microscopic size. . . . These ultramicroscopic particles (ultramicros) have the same significance for colloidal solutions as the isolated molecules have for crystalloidal solutions.³

¹ This is no longer correct, as we shall see.

² GRAHAM, T., *Phil. Trans.*, pp. 183-224, 1861. Reprinted in "Chemical and Physical Researches," p. 553, Edinburgh, 1876.

³ ZSIGMONDY, R., "Kolloidchemie," 2d ed., Leipzig, 1918.

The idea that the ultimate unit of the colloidal solution is not the molecule or ion of the solute, but an aggregate, induced colloid chemists to propose a new type of chemistry in which the laws of classical chemistry were replaced by laws peculiar to colloid chemistry. It seemed improbable to them that the stoichiometrical laws of classical chemistry should hold for colloidal solutions in which the ultimate units were larger aggregates of molecules, since they argued that only the surface of such aggregates should be capable of reacting with other substances. The stoichiometrical relations valid in classical chemistry were, as a consequence, replaced in colloid chemistry by an empirical formula, namely, Freundlich's so-called adsorption formula, which was supposed to account for surface action.¹ Recent investigations by Langmuir² have furnished the proof that Freundlich's adsorption formula does not hold for the reaction of gases with mica, glass, and platinum possessing a smooth surface, and Langmuir was able to show that the forces which act in these cases are the purely chemical forces of primary or secondary valency. Like most empirical formulas the adsorption formula may hold within a limited range of observations, but not throughout the whole range of variation, and Langmuir states that this was also true for the adsorption formula in his experiments.

John A. Wilson and Wynnaretta H. Wilson³ have made a most important contribution towards the question of the applicability of the adsorption formula to colloidal problems, in which they were also led to a rejection of the adsorption formula and to the adoption of a purely chemical interpretation. Their discussion is based on the experiments of Procter and Wilson on gelatin and the facts to be given in this book fully support their skeptical attitude towards the adsorption formula. T. B. Robertson⁴ and E. A. Fisher⁵ have also expressed doubts as to the value of the adsorption formula.

¹ FREUNDLICH, H., "Kapillarchemie," Leipsic, 1909.

² LANGMUIR, I., *J. Am. Chem. Soc.*, vol. 40, p. 1361, 1918.

³ WILSON, J. A. and WILSON, W. H., *J. Am. Chem. Soc.*, vol. 40, p. 886, 1918.

⁴ ROBERTSON, T. B., "The Physical Chemistry of the Proteins," New York, London, Bombay, Calcutta, and Madras, 1918.

⁵ FISHER, E. A., *Trans. Faraday Soc.*, vol. 17, part 2, p. 305, 1922.

Even if we assume that the protein solutions contain no free protein ions or molecules—which is contradicted by the experiments on membrane potentials and osmotic pressure to be discussed later—such an assumption does not lead to the idea that chemical reactions occur only at the surface of the micellæ, for the simple reason that solid gels of proteins (*e.g.*, of gelatin) are easily permeable to acids, alkalies, and salts or to crystalloids in general. Chemical reactions are therefore not restricted to the surface of protein micellæ.

While a number of authors, like Bugarszky and Liebermann,¹ Osborne,² Robertson,³ Pauli,⁴ and others, assumed that the reactions of proteins are purely chemical, this assumption could not be proved conclusively until the modern methods of measuring the hydrogen ion concentration of protein solutions were developed by Friedenthal, Sørensen,⁵ Michaelis,⁶ Clark,⁷ and their collaborators. On the basis of these methods it was easy to demonstrate the purely stoichiometrical character of the combination of proteins with acids and alkalies.

Thus it was proved that gelatin combines with acids only when the hydrogen ion concentration of the solution is above a certain critical point, namely, greater than $N/50,000$ (or $\text{pH} = 4.7$).⁸ At hydrogen ion concentrations above $N/50,000$, H_3PO_4 dissociates as a monobasic acid. Hence, if gelatin combines stoichiometrically with acids, it should require three times as many cubic centimeters of $0.1\ N\ \text{H}_3\text{PO}_4$ as it requires cubic centimeters of $0.1\ N\ \text{HCl}$ or HNO_3 to bring 1 gm. of gelatin in a 100-c.c. solution from a hydrogen ion concentration of $N/50,000$ to that of,

¹ BUGARSZKY, S. and LIEBERMANN, L., *Arch. ges. Physiol.*, vol. 72, p. 51, 1898.

² OSBORNE, T. B., "Die Pflanzenproteine," *Ergebnisse Physiol.*, vol. 10, p. 47, 1910.

³ ROBERTSON, T. B., "The Physical Chemistry of the Proteins," New York, London, Bombay, Calcutta, and Madras, 1918.

⁴ PAULI, W., *Fortschritte naturwiss. Forschung*, vol. 4, p. 223, 1912; "Kolloidchemie der Eiweisskörper," Dresden and Leipsic, 1920.

⁵ SØRENSEN, S. P. L., see Bibliography given in CLARK, W. M., "The Determination of Hydrogen Ions," Baltimore, 1920.

⁶ MICHAELIS, L., "Die Wasserstoffionenkonzentration," Berlin, 1914.

⁷ CLARK, W. M., "The Determination of Hydrogen Ions," Baltimore, 1920.

⁸ LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 85, 1920-21; *Science*, vol. 52, p. 449, 1920; *J. chim. phys.*, vol. 18, p. 283, 1920.

e.g., $N/1,000$. The strong acid H_2SO_4 dissociates, however, in this range of hydrogen ion concentration as a dibasic acid and hence it should require as many cubic centimeters of $0.1\ N\ H_2SO_4$ as it requires cubic centimeters of $0.1\ N\ HCl$ to bring the same 1 per cent solution of gelatin from a hydrogen ion concentration of $N/50,000$ to one of $N/1,000$. Titration experiments proved the correctness of these and similar conclusions, not only in the case of gelatin but also of other proteins, namely, crystalline egg albumin, casein, edestin, and serum globulin, thus leaving no doubt that proteins combine with acids or alkalies according to the stoichiometrical laws of general chemistry.¹

It was merely an unfortunate historical accident that the colloidal behavior of proteins was investigated before the convenient methods of measuring the hydrogen ion concentration were developed; otherwise, we should probably never have heard of the idea that the chemistry of colloids differs from the chemistry of crystalloids, at least as far as the proteins are concerned. It was this methodical error of not measuring the hydrogen ion concentration of colloidal solutions and of gels which prevented the development of an exact theory of colloidal behavior and which gave rise to the pessimistic statement of Zsigmondy quoted in the preface.

The reason that measurements of the hydrogen ion concentration are paramount for the understanding of the chemical and physical behavior of the proteins lies in the fact that proteins are amphoteric electrolytes capable of forming ionizable salts with acids as well as with alkalies, according to the hydrogen ion concentration. When the hydrogen ion concentration exceeds a certain critical value (which varies for different proteins), the protein behaves as if it were a base, like NH_3 , capable of forming salts with acids; while when the hydrogen ion concentration of the solution is below this critical value, the protein behaves as if it were a fatty acid, *e.g.*, CH_3COOH , capable of forming salts with bases. At the critical value of the hydrogen ion concentration the protein can practically combine neither with an acid nor a base nor a neutral salt.² This critical hydrogen ion concen-

¹ LOEB, J., *J. Gen. Physiol.*, vol. 3, pp. 85, 547, 1920-21. HIRCHCOCK, D. I., *J. Gen. Physiol.*, vol. 4, pp. 597, 733, 1921-22; vol. 5, p. 35, 1922-23.

² LOEB, J., *J. Gen. Physiol.*, vol. 1, pp. 39, 237, 1918-19; *Science*, vol. 52, p. 449, 1920; *J. chim. phys.*, vol. 18, p. 283, 1920.

tration is called the "isoelectric" point of the protein. Moreover, we shall see that the fraction of 1 gm. of originally isoelectric protein in a 100-c.c. solution capable of combining with an acid or alkali is also a definite function of the hydrogen ion concentration.

2. THE ISOELECTRIC POINT OF PROTEINS

The conception of the "isoelectric point" of proteins was introduced before its chemical meaning was recognized and it attracted attention because it was connected with the precipitation of colloids, a phenomenon on which the interest of a number of investigators had been focussed. The conception of the isoelectric point of proteins, which is due to W. B. Hardy,¹ must be considered as the starting point for the physical chemistry of proteins. This author found in 1899 that white of egg diluted with eight or nine times its volume of distilled water, filtered, and boiled, when put into an electrical field migrated in an opposite direction according to whether the reaction of the fluid was acid or alkaline. When the fluid had an alkaline reaction, the particles moved in an electrical field from the cathode to the anode; when the fluid was acid, the direction of the motion of the particles was the reverse, namely, from the anode to the cathode; when the fluid was neutral, the movement of the particles under the influence of a current was so slight that it was difficult to detect.

I have shown that the heat-modified proteid is remarkable in that its direction of movement [in an electric field] is determined by the reaction, acid or alkaline, of the fluid in which it is suspended. An immeasurably minute amount of free alkali causes the proteid particles to move against the stream, while in the presence of an equally minute amount of free acid the particles move with the stream. In the one case, therefore, the particles are electronegative, in the other they are electropositive. Since one can take a hydrosol in which the particles are electronegative and, by the addition of free acid, decrease their negativity, and ultimately make them electropositive, it is clear that there exists some point at which the particles and the fluid in which they are immersed are isoelectric.

The isoelectric point is found to be one of great importance. As it is neared the stability of the hydrosol diminishes until, at the isoelectric point, it vanishes, and coagulation or precipitation occurs, the one or the

¹ HARDY, W. B., *Proc. Roy. Soc.*, vol. 66, p. 110, 1900.

other according to whether the concentration of the proteid is high or low, and whether the isoelectric point is reached slowly or quickly, and without or with mechanical agitation.

In a preliminary note¹ on his work on globulins published in 1903 Hardy gives an interpretation of the influence of H and OH ions on the direction of migration of protein particles in an electrical field which was destined to play an important rôle in colloid chemistry, since it suggested to the later workers that the H and OH ions produced their influence on the electrical charge of the protein particles through a process of preferential adsorption.

The properties of globulins in solution seem to justify the following view: *They are not embraced by the theorem of definite and multiple proportions. Therefore they are conditioned by purely chemical forces only in a subsidiary way.* A precipitate of globulin is to be conceived not as composed of molecular aggregates but of particles of gel. I have shown elsewhere that gelation and precipitation of colloidal solutions are continuous processes. These particles of gel when suspended in a fluid containing ions are penetrated by those ions. Let the fundamental assumption be that the higher the specific velocity of an ion the more readily it will become entangled within the colloidal particle. Then, as H and OH ions have by far the highest specific velocity, the colloidal particle will entangle an excess of H ions in acid and thereby acquire a + charge, and of OH ions in alkali and thereby acquire a - charge. These charges will decrease the surface energy of the particle and thereby lead to changes in their average size.

Perrin² adopted the idea that H and OH ions confer their electrical charge to colloidal particles on account of their relatively large velocity of migration, whereby they were readily adsorbed by the colloidal particle. The hypothesis of a preferential adsorption of H and OH ions by colloidal particles has since played a great rôle in colloid chemistry.

In 1904 the writer of this volume offered, instead of this colloidal, a purely chemical view of the significance of the isoelectric point and of the cause of the influence of acids and alkalies on the direction of the migration of the colloidal particles.³

¹ HARDY, W. B., *J. Physiol.*, vol. 29, p. xxix, 1903.

² PERRIN, J., *J. chim. phys.*, vol. 2, p. 601, 1904; vol. 3, p. 50, 1905;

"Notice sur les Titres et Travaux Scientifiques," Paris, 1918.

³ LOEB, J., *Univ. Calif. Pub., Physiology*, vol. 1, p. 149, 1904.

It seems to the writer, however, that a different view of these phenomena is possible, whereby they appear in harmony with the view of electrolytic origin of the charges of colloids. The proteids are known to be amphoteric in their reaction. If they be slightly dissociable, they will send H as well as OH ions into the solution. When the particles send more H ions than OH ions into the solution, they will have a negative charge, while they will have a positive charge when more OH ions are given off than H ions. If acid is added to the solution in sufficient concentration, the amphoteric colloidal particle will send more OH ions into the solution than H ions and hence will assume a positive charge. The reverse will be the case in an alkaline solution. It harmonizes with this idea that, as Hardy found, neutral salts, do not influence the sign of the electrical charge of the globulins.

In his famous paper on "Colloidal Solution," published in 1905, Hardy abandons the physical view which he expressed in 1903 and adopts "a frankly chemical standpoint:"

Globulin, therefore, is an amphoteric substance and its acid function is much stronger than its basic function. As an acid it is strong enough to form salts readily with bases so weak as aniline, glycoceoll, and urea; acting as a base it forms salts with weak acids, such as acetic, and boracic acids, which are very unstable in presence of water.¹

Though one may speak of the colloid particles as being ionic in nature, they are sharply distinct from true ions in the fact that they are not of the same order of magnitude as are the molecules of the solvent, the electric charge which they carry is not a definite multiple of a fixed quantity and one cannot ascribe to them a valency, and their electrical relations are those which underlie the phenomena of electrical endosmose. To such ionic masses I would give the name "pseudo-ions" and I propose to treat globulin solutions from the standpoint of a hypothesis of pseudo-ions.²

And in 1909 Wood and Hardy³ express the view that proteins react with acids and alkalies to form salts, but the reactions are not precise, an indefinite number of salts of the form $(B)_nBHA$ being formed, where the value of n is determined by conditions of temperature and concentration, and of inertia due to electrification of internal surfaces within the solution.

¹ HARDY, W. B., *J. Physiol.*, vol. 33, p. 251, 1905-06; *Proc. Roy. Soc.*, vol. 79, p. 413, 1907.

² HARDY, W. B., *J. Physiol.*, vol. 33, pp. 256-257, 1905-06.

³ WOOD, T. B. and HARDY, W. B., *Proc. Roy. Soc.*, vol. 81, p. 38, 1909.

There are three different elements in Hardy's view, which must be separated: First, the idea that proteins are capable of forming salts with acids and alkalies is undoubtedly correct. Second, that the reactions between proteins and acids or alkalies are not stoichiometric, an idea that seems no longer tenable. Third, that the ultimate units of protein in solution are not isolated molecules but larger aggregates which are kept in suspension by their electrical charges. We shall return to the discussion of this third idea in the next paragraph.

When the methods of measuring the hydrogen ion concentration had been developed by H. Friedenthal and by Sørensen, it became possible to determine the isoelectric point of genuine proteins. This was done most extensively by Michaelis and his collaborators in 1910. Michaelis used the same method of migration of the particles in an electrical field which had been used by Hardy in his experiments on denatured protein particles. The isoelectric point is, according to Michaelis, that hydrogen ion concentration at which the particles migrate neither to the anode nor to the cathode. The following figures give the hydrogen ion concentrations defining the isoelectric points of different proteins as determined by Michaelis.¹

Genuine serum albumin.....	2	$\times 10^{-6}N$
Genuine serum globulin.....	4	$\times 10^{-6}N$
Oxyhemoglobin.....	1.8	$\times 10^{-7}N$
Gelatin.....	2	$\times 10^{-6}N$
Casein.....	2	$\times 10^{-5}N$

According to Sørensen the isoelectric point of crystalline egg albumin is near that of serum albumin (namely, at a pH of 4.8).²

We shall denote in this book the hydrogen ion concentration by Sørensen's logarithmic symbol, pH; *e.g.*, the concentration $2 \times 10^{-6}N = 10^{-4.7}N$ is written merely pH 4.7, the minus sign being omitted.

If we assume that the ultimate units of a protein solution are as a rule isolated protein molecules or ions which react stoichiometrically with acids and alkalies, forming highly dissociable

¹ MICHAELIS, L., "Die Wasserstoffionenkonzentration," p. 54 ff, Berlin, 1914.

² SØRENSEN, S. P. L., "Studies on Proteins," *Compt. rend. trav. lab. Carlsberg*, vol. 12, Copenhagen, 1915-17.

metal proteinates or protein-acid salts, we may define the isoelectric point of a protein as that hydrogen ion concentration in which the protein exists practically in a non-ionogenic (or non-ionized) condition being able to form practically neither metal proteinate nor protein-acid salt. We shall see that this theoretical result leads to a simple practical method of preparing proteins entirely or practically free from ionogenic impurities.

3. COLLOIDAL SUSPENSIONS AND CRYSTALLOIDAL SOLUTIONS

Graham had suggested the distinction between colloidal and crystalloidal substances, but it was found later that one and the same substance, *e.g.*, NaCl, may behave when in solution either as a crystalloid or as a colloid. It then was proposed to drop the distinction between colloidal and crystalloidal *substances* and to distinguish between the colloidal and the crystalloidal *state of matter*. The reasons are summed up in the following quotation from Burton:

Modern work has shown that it is incorrect to speak of colloidal substances as a particular class. Krafft has observed that the alkali salts of the higher fatty acids—stearate, palmitate, oleate—dissolve in alcohol as crystalloids with normal molecular weights, but in water they are true colloids. The reverse is true of sodium chloride; Paal found that the latter gave a colloidal solution in benzol, while, of course, it gives a crystalloidal solution in water (Karczag). More recently, von Weimarn has demonstrated, by the preparation of colloidal solutions of over two hundred chemical substances (salts, elements, etc.), that, by proper manipulation, almost any substance which exists in the solid state can be produced in solution, either as a colloid or as a crystalloid; and that, as shown by many other workers, in some cases it is merely a matter of the concentration of the reacting components whether one gets crystalloidal or colloidal solutions.

Consequently, we now speak of matter being in the colloidal state rather than of certain substances as colloids—the essential characteristic of the colloidal state being that the substance will exist indefinitely as a suspension of solid (or, in some cases, probably liquid) masses of very small size in some liquid media, *e.g.*, water, alcohol, benzol, glycerin, etc. According to the medium employed, the resulting solutions or suspensions are called, after Graham, hydrosols, alcosols, benzosols, glycersols, etc.¹

¹ BURTON, E. F., "The Physical Properties of Colloidal Solutions," 2d ed., pp. 8-9, London, New York, Bombay, Calcutta, and Madras, 1921.

The chief distinction between colloids and crystalloids, or rather between the colloidal and crystalloidal state, lies in the nature of the forces by which the two kinds of substances are kept in solution (in water or in some other solvent). The forces which determine the stability of crystalloids in solution are, according to Langmuir or Harkins, forces of a chemical nature, the molecules of solute and solvent being attracted to each other by chemical affinity—so-called secondary valency forces. It may be stated, in general, that as long as the forces of attraction between the molecules of the solvent and the solute are sufficiently large, the solution will be stable. When these forces become too small, aggregates of molecules may be formed, but these aggregates may remain in suspension, provided a potential difference is established between each particle and the solvent. In this case, we have no longer a true solution but a colloidal solution, *i.e.*, a suspension. The forces which guarantee the stability of colloidal solutions, *i.e.*, of suspensions of fine solid particles or of fine emulsions of pure oil in water, are forces of electrostatic repulsion due to the existence of a double electrical layer at the interface between each particle and the water. As long as these potential differences of the double electrical layer are sufficiently high, the particles upon approaching each other will repel each other electrostatically and this will prevent the coalescence of the finer into larger particles, which settle more rapidly under the influence of gravity. Jevons¹ first pointed out that the electrostatic repulsion between the particles due to these electrical charges might be the force which prevents the fine suspended particles from coalescing and settling, but it is the merit of Hardy to have proved this idea by showing that suspensions of fine particles of denatured (boiled) white of egg are no longer stable at the isoelectric point where their charges are zero. When the charge is annihilated or sufficiently diminished, "the adhesion, or 'idioattraction,' as Graham called it, of the colloid particles for each other makes them cohere when they come together."² Of course, if the forces of cohesion between the molecules of particles are too small, the particles may remain in suspension, even if they are not charged at all.

¹ JEVONS, W., *Trans. Manc. Phil. Soc.*, p. 78, 1870.

² WOOD, T. B. and HARDY, W. B., *Proc. Roy. Soc.*, vol. 81, p. 41, 1909.

Northrop and De Kruif have shown this to be true under certain conditions for bacterial suspensions.¹

On the other hand, suspended particles can generally be flocculated with the aid of salts, even if the particles originally carried a high charge. In this case Hardy assumes correctly that the addition of salt lowers the potential difference between the colloidal particle and the solvent, and this assumption was confirmed by measurements of the charges by numerous authors.²

Schulze,³ and later Linder and Picton,⁴ in studying the coagulation of suspensions of arsenious sulphide by salts found that the coagulative power depended on the valency of the metal of the salt, increasing rapidly with the valency of the metal, and Hardy showed that quite generally flocculation (coagulation, agglutination) of the suspended particles was due to that ion of the salt which had a charge opposite to that of the suspended particles.

A second equally striking fact brought out by these investigations was that the concentration of the salt required for the precipitation of these suspensions was always low, especially when the ion bearing the opposite charge to that of the suspended particles was plurivalent.

The question now arises: Which of the two kinds of forces, electrostatic repulsion or secondary valency forces, determines the solution of genuine proteins in water? The decision of this question can be rendered with the aid of the following criterion: When the forces which keep a protein in solution are the forces of attraction between the molecules of solute and solvent (*i.e.*, the forces of secondary valency in Langmuir's definition), high concentrations of a salt are required for precipitation and the sign of charge of the precipitating ion need have no relation to the sign of charge of the protein particle. When, however, these forces of secondary valency between molecules of solution and solvent are small the particles may nevertheless remain in suspension with the aid of electrical double layers

¹ NORTHROP, J. H. and DE KRUIF, P. H., *J. Gen. Physiol.*, vol. 4, p. 639, 1921-22.

² BURTON, E. F., "The Physical Properties of Colloidal Solutions," 2d ed., London, New York, Bombay, Calcutta, and Madras, 1921.

³ SCHULZE, H., *J. prakt. Chem.*, vol. 25, p. 431, 1882; vol. 27, p. 320, 1883; vol. 32, p. 390, 1884.

⁴ LINDER, S. E., and PICTON, H., *J. Chem. Soc.*, vols. 61, 67, 71, and 87.

surrounding each particle. When this is the case low concentrations of salts suffice to precipitate the protein and the effective ion of the salt has a sign of charge opposite to that of the protein particle. When this criterion is applied to protein solutions, it is found that certain proteins, *e.g.*, denatured white of egg, are kept in colloidal suspension by double electrical layers, while certain other proteins, such as genuine crystalline egg albumin or gelatin, are kept in solution by the same forces which determine the stability of crystalloidal solutions.

The fact that crystalline egg albumin or gelatin requires high concentrations of salt for precipitation led some authors to suggest that these proteins form emulsions instead of suspensions. It became customary to distinguish between two types of colloidal solutions, suspensoids (*e.g.*, clay or graphite), which required low concentrations of salts for precipitation, and emulsoids, which required high concentrations of salts for precipitation. Other terms were hydrophilic or lyophilic colloids for emulsoids or hydrophobic or lyophobic colloids for suspensoids. Genuine proteins were supposed to be emulsoids or hydrophilic colloids. Unfortunately for this assumption, it was shown by Powis¹ that true emulsions of oil in water are flocculated by the same low concentrations of salts as are the suspensions of solid particles. The so-called emulsoids, or hydrophilic colloids, behave like crystalloids in regard to solubility, but not like emulsions.

Proteins are composed of amino-acids in peptide linkage. Each amino-acid is a crystalloid and there is no *a priori* reason why the nature of the forces which drag the amino-acids into solution should be necessarily lost when they are linked into polypeptides. There is, however, a difference between the solubility of different amino-acids, some, like glycine or alanine, being highly soluble in water, while others, like tyrosine, are sparingly soluble. According to the nature and perhaps the mode of linkage of the amino-acids constituting the different proteins, there must exist differences in the solubility of the proteins. When the non-soluble amino-acids or non-soluble groups prevail in a protein, it may happen that the attraction between water and the protein becomes so small that only double

¹ POWIS, F., *Z. physik. Chem.*, vol. 89, pp. 91, 179, 186, 1914-15.

electrical layers can keep the particles in solution. It would, however, be wrong to infer from this that solutions of all proteins behave in this way.

The idea that genuine proteins may form crystalloidal solutions meets with two difficulties, which, however, are not real. The one is due to the fact that many genuine proteins, *e.g.*, gelatin, are sparingly soluble at the isoelectric point. Since at the isoelectric point the particles do not migrate in a galvanic field this was interpreted to mean that electrical double layers kept the isoelectric gelatin in solution. We shall see that it means only that non-ionized protein is less soluble than ionized protein.¹ Michaelis had pointed out that the crystalloidal amino-acids also possess minimal solubility at the isoelectric point.² The second difficulty was found in the fact that a gelatin solution sets to a gel. This seemed to contradict the assumption that gelatin is kept in solution by the affinity between certain groups of its molecule and water. The following suggestion may relieve this difficulty. In the large protein molecule there are certain groups (*e.g.*, COOH and NH₂) with a high affinity for water (which we will call "aqueous" groups), while there are other groups with low affinity for water and high affinity for each other (which we will call "oily" groups). While the oily groups are not able to overcome the forces with which the aqueous amino or carboxyl groups drag the molecule of protein into the water, they may give rise to a chain or network formation between neighboring protein molecules or ions, whenever it happens that oily groups of two different molecules touch each other. This is probably the mode of origin of the solid gel of gelatin in water. The jelly formation of gelatin seems to differ from the case of precipitation in this, that, while in precipitation even the aqueous groups or atoms of a molecule lose their affinity for water, in the case of jelly formation the affinity of the aqueous groups for water is practically unimpaired, the molecules adhering to each other only by certain oily groups of the large molecule. When

¹ LOEB, J., *Arch. Néerland. physiol.*, vol. 7, p. 510, 1922. COHN, E. J., *J. Gen. Physiol.*, vol. 4, p. 697, 1921-22. COHN, E. J., and HENDRY, J. L., *J. Gen. Physiol.*, vol. 5, p. 521, 1922-23.

² MICHAELIS, L., "Die Wasserstoffionenkonzentration," pp. 41-44, Berlin, 1914.

such a jelly is formed the relative distance of the gelatin molecules from each other remains, on the average, the same as it was in the solution; and the forces of adhesion between water and the aqueous groups of the gelatin molecules also remain unaltered; what is altered is only the orientation of the individual molecules of gelatin towards each other, since they now touch each other with their oily groups.

There seems no reason for assuming that the forces which drag molecules of certain genuine proteins, such as crystalline egg albumin or gelatin, into solution differ from the forces which drag crystalloids into solution.

4. THE HOFMEISTER ION SERIES

The idea that all solutions of genuine proteins are diphasic systems in which the individual particles are kept in solution by an electrical double layer was linked with the assumption of the preferential adsorption of ions by the particles. This assumption of a preferential adsorption of ions by particles seemed to be supported by experiments intended to show that different ions of the same sign and valency influenced the physical properties of proteins in an entirely different way. These experiments were initiated by Hofmeister.¹ He and his followers observed that the relative effects of anions on the precipitation, the swelling, and other properties of proteins seemed very definite and that the anions could be arranged apparently in definite series according to their relative efficiency, the order being independent of the nature of the cation. Similar series were also found for the cations, though these series seemed to be less definite.

In an excellent summary of the statements in the literature given by Höber,² the series are as follows:

At "neutral" reaction the anions of salts are stated to influence swelling, osmotic pressure, or viscosity in the following order:

SO_4 , tartrate, citrate < acetate < Cl < Br, NO_3 < I < CNS,

where the swelling is said to be a maximum in CNS and a minimum in SO_4 . Above a certain concentration the sulphates,

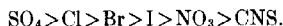
¹ HOFMEISTER, F., *Arch. expl. Path. Pharmacol.*, vol. 24, p. 247, 1888; vol. 25, p. 1, 1888-89; vol. 27, p. 395, 1890; vol. 28, p. 210, 1891.

² HÖBER, R., "Physikalische Chemie der Zelle und der Gewebe," 5th ed., part 1, p. 267, Leipsic, 1922.

tartrates, and citrates are said to cause a shrinkage of the gel of gelatin, and acetate is said to act in the same sense, but less strongly; while the other anions are believed to cause an increasing swelling of the gel of the same concentration.

As far as the action of cations is concerned, according to Höber's summary "the differences in the effect of cations are less marked; it might be possible to propose the series $\text{Li} < \text{Na} < \text{K}$, NH_4 ; then follow the alkali earths with Mg in a position between."

As far as osmotic pressure effects are concerned, Lillie's experiments are quoted by Höber, according to which anions depress the osmotic pressure of neutral solutions of egg albumin in the following order:



In the development of these Hofmeister series, a serious error has been committed, namely, the neglect of measuring the hydrogen ion concentrations of the protein solutions and protein gels and of comparing the effect of ions at the same pH of the protein solution or protein gel. As a consequence, effects which are due only to variations in the hydrogen ion concentration were erroneously ascribed to the chemical nature of the anion. If the hydrogen ion concentrations are duly measured with the hydrogen electrode and properly taken into consideration, it is found that certain properties of proteins are influenced only by the valency of the ion and not by its chemical nature; and that, further, in these cases only that ion has any effect the sign of charge of which is opposite to that of the protein ion.

This is true for those properties of proteins which are specifically colloidal, namely, osmotic pressure, swelling, and a certain type of viscosity.

Thus we shall see in this book that NaCl , NaBr , NaI , NaNO_3 , Na acetate, Na propionate, and Na lactate depress the osmotic pressure and viscosity of gelatin chloride solutions and the swelling of a gel of gelatin chloride of a definite pH quantitatively alike, and that the Hofmeister anion series are fictitious; it will further be shown that the effect of LiCl , NaCl , KCl , CaCl_2 , and LaCl_3 on these properties of gelatin chloride is also identical for the same concentration of Cl ions and the same pH; and that the Hofmeister cation series are in this case also fictitious.

It will be shown likewise that all divalent anions have a much stronger effect on the three properties of protein chloride mentioned than the monovalent anions; and that the divalent anions act alike regardless of their chemical nature.

All these facts prove that only the valency, and not the chemical nature of an ion, influences such properties of proteins as osmotic pressure, swelling, and a certain type of viscosity. This fact is of capital importance because it definitely settles the origin of the colloidal behavior of proteins. We know only one group of properties which are influenced by the valency but not by the chemical nature of ions, namely, properties depending on membrane equilibria, to which we shall return later.

The fact that the chemical nature of ions has no effect on the three properties of protein mentioned and that the Hofmeister ion series are only the results of a methodical error creates a difficulty for the adsorption hypothesis to which we shall return at the end of Chap. VIII.

We have thus far spoken only of the influence of ions on such specifically colloidal properties of proteins as osmotic pressure, swelling, and viscosity. We may add that the crystalloidal properties, such as solubility and cohesion, are in all probability affected not only by the valency but also by the chemical nature of ions. If this is true, "Hofmeister series" might probably be found for such crystalloidal properties.

5. THE AGGREGATION HYPOTHESIS

It was perhaps not very fortunate for the development of a theory of colloids that the attention of investigators was at first focussed especially on the phenomena of precipitation. Since precipitation is due to an aggregation of particles, it overemphasized the significance of aggregate formation. This led, as we have seen, to the erroneous idea that proteins do not combine stoichiometrically with other compounds, since aggregates were assumed to react only at their surface—an assumption which, as already stated, is not warranted in the case of proteins, since protein gels are freely permeable to crystalloids. It led, however, to another equally fatal idea, that this aggregate formation would explain all the colloidal phenomena. Thus, when R. S. Lillie¹ made the

¹ LILLIE, R. S., *Am. J. Physiol.*, vol. 20, p. 127, 1907-08.

important observation that neutral salts depress the osmotic pressure of gelatin solutions, it seemed natural to explain this fact from the precipitating action of salts, by assuming that the addition of salt caused an aggregation of gelatin molecules or ions into larger aggregates.¹ This would lead to a diminution of the number of particles in solution. But it was also found that the addition of salts depresses the viscosity of protein solutions and the swelling of solid proteins. We shall see later that the formation of aggregates out of isolated protein molecules or ions increases the viscosity of a gelatin solution.² Hence, if the addition of a salt to a protein solution diminishes its osmotic pressure by causing an increased formation of aggregates, the same addition of salt should increase the viscosity of such a solution. In reality, however, the reverse happens, the viscosity of the solution being decreased, instead of being increased by the addition of salt.

There is a second difficulty. The salting out of gelatin requires high concentrations of salt and the effective ion bears no relation to the charge of the protein; while the depressing action of salts on the osmotic pressure of gelatin solutions is, as we shall see, exclusively due to that ion of the salt which carries the opposite charge to that of the protein; and, moreover, low concentrations of the salt suffice to annihilate the osmotic pressure of protein solutions.

The dispersion or aggregation hypothesis cannot be tested quantitatively and at no time in the history of science has it been possible to find an explanation for complicated phenomena by merely qualitative and non-mathematical speculations.

6. PAULI'S HYDRATION THEORY

Laqueur and Sackur,³ in studying the influence of the addition of different quantities of NaOH to a given mass of casein, assumed correctly that the two substances combined to form sodium caseinate. The viscosity of the sodium caseinate solution was high and it varied in a peculiar way with the quantity of

¹ ZSIGMONDY, R., "Kolloidchemie," 2d ed., Leipsic, 1918.

² LOEB, J., *J. Gen. Physiol.*, vol. 4, p. 97, 1921-22.

³ LAQUEUR, E. and SACKUR, O., *Beitr. chem. Physiol. Pathol.*, vol. 3, p. 193, 1903.

NaOH added to the casein. When little NaOH was added, the viscosity increased at first with an increase in the quantity of the NaOH added, until a maximum was reached, when the addition of more NaOH again diminished the viscosity. This, again, is a fundamental fact which has since been confirmed for the influence of acids and alkalies not only upon the viscosity but also upon other properties of proteins, and which holds not only for casein but apparently for all proteins.

Laqueur and Sackur explained their results on the basis of Reyher's¹ experiments on the viscosity of solutions of fatty acids. Reyher had found that the viscosity of solutions of salts of the fatty acids is greater than that of solutions of fatty acids themselves; and, since the salts of the fatty acids undergo electrolytic dissociation to a much greater extent than the acids, it was assumed that the increase in viscosity is determined chiefly by the ionization. Laqueur and Sackur made the same assumption for the casein solutions, attributing the high viscosity of casein solutions to the casein ions, and they support their assumption by the fact that the addition of little NaOH to casein at first increases the viscosity until a maximum is reached and that the addition of more NaOH diminishes the viscosity again. A diminution of viscosity could also be produced by the addition of neutral salt to the solution of Na caseinate. Laqueur and Sackur assume that this drop in the viscosity is caused by a lowering of the degree of electrolytic dissociation of the Na caseinate by the Na ion of the NaOH or NaCl added in excess.

The idea that the viscosity of a protein solution depends primarily upon the protein ion was accepted by W. Pauli,² who made the additional hypothesis that each protein ion is hydrated; i.e., that each individual protein ion is surrounded by a considerable shell of water. Pauli worked with blood albumin which had been freed from salts by a dialysis continued for several weeks. When he added acid to water-soluble albumin, the viscosity increased first from 1.0623 for the pure albumin solution to 1.2937 when the concentration of HCl added to the albumin solution was 0.017 N. When the HCl concentration was

¹ REYHER, R., *Z. physik. Chem.*, vol. 2, p. 744, 1888.

² PAULI, W., *Fortschritte naturwiss. Forschung*, vol. 4, p. 223, 1912; "Kolloidchemie der Eiweisskörper," Dresden and Leipsic, 1920.

increased to 0.05 N, the viscosity was only 1.1667. The following figures give the data, according to Pauli:

Concentration of HCl.....	0.0 N	0.005 N	0.01 N	0.012 N	0.017 N	0.02 N	0.03 N	0.04 N	0.05 N
Viscosity.....	1.0623	1.2555	1.233	1.274	1.2937	1.2770	1.224	1.1822	1.1667

Pauli assumed that the protein ions are surrounded by a jacket of water, while the non-ionized molecules of protein he assumed not to be hydrated. Addition of a little HCl to isoelectric albumin would cause the transformation of non-ionized albumin into albumin chloride which is highly ionized and hence assumed to be highly hydrated; the more acid is added the more albumin chloride and the more hydrated albumin ions should be formed. Hence the viscosity should at first increase with the quantity of acid added until a point is reached where the addition of more acid represses the degree of electrolytic dissociation of the albumin chloride on account of the high concentration of the Cl ion common to both protein chloride and HCl.

If we intend to use these ideas for the explanation of the influence of the valency of ions on the physical properties of proteins, we are compelled to assume that the degree of electrolytic dissociation of gelatin salts with bivalent ions is lower than that of gelatin salts with monovalent ions. Since, *e.g.*, the viscosity of gelatin chloride solutions is considerably higher than the viscosity of gelatin sulphate solutions of the same hydrogen ion concentration and the same concentration of originally isoelectric gelatin, we should have to conclude that the degree of electrolytic dissociation of gelatin sulphate is considerably less than that of gelatin chloride.

The writer put this theory to a test by measuring the electrical conductivity of solutions of different gelatin salts at different pH, with the result that the parallelism between the concentration of protein ions and the physical properties of proteins demanded by Pauli's theory could not be demonstrated (see Chap. IX). Lorenz,¹ Born,² and other authors have recently reached the

¹ LORENZ, R., *Z. Elektrochem.*, vol. 26, p. 424, 1920.

² BORN, M., *Z. Elektrochem.*, vol. 26, p. 401, 1920.

conclusion that the idea of a hydration of ions is not tenable in the case of polyatomic ions.¹

The increase in viscosity of certain protein solutions through the addition of acid or alkali to isoelectric proteins is caused by the ionization of proteins, but the connection is not the direct one suggested by Laqueur and Sackur, but, in the case of solutions of gelatin or casein, an indirect one, due to the rôle of protein ions in the establishment of a Donnan equilibrium. The ideas of Reyher and Pauli may, however, find an application to another type of viscosity which is found in crystalloids, *e.g.*, amino-acids.

7. RECAPITULATION

If we now recapitulate the history of this subject, we notice that in the colloidal literature proteins were assumed to combine not stoichiometrically but by adsorption. Because proteins are amphoteric electrolytes and their salts are strongly hydrolyzed, it is necessary to measure the hydrogen ion concentration of protein solutions with the hydrogen electrode before any conclusions can be drawn concerning the nature of the combination of proteins. If this advice is followed, it is found that proteins react stoichiometrically with acids and alkalies, and that there is no difference between the chemistry of proteins and that of crystalloids.

It was further argued that solutions of genuine proteins in water are always diphasic systems in which the particles of protein are kept in solution on account of electrical double layers, due to an alleged preferential adsorption of ions by particles. It was shown that certain proteins are kept in solution by the same forces which determine the solution of crystalloids, *e.g.*, the amino-acids from which the proteins are built up. The forces which keep such genuine proteins in solution do not differ from the forces which keep crystalloids, like amino-acids, in solution.

Finally, the belief in the reality of the Hofmeister ion series lent further support to the belief in the adsorption theory; yet it is possible to show that these series for swelling, osmotic

¹ The term "hydration" is often used in colloid chemistry in a vague way to designate such phenomena as the swelling of proteins, which is a purely osmotic phenomenon. It is obvious that it can only lead to confusion if the term "hydration" is used for osmotic pressure. In this volume the term "hydration" is only used in the sense of Kohlrausch and Pauli.

pressure, and viscosity, were also based on a methodical error, namely, the failure to measure the hydrogen ion concentration of the solutions. It is, therefore, obvious that the so-called colloid chemistry of proteins is a system of errors based on inadequate and antiquated methods of experimentation.

The question then arises: Are there any peculiarities in the behavior of proteins which are not found in crystalloids? To this the answer must be given that such peculiarities exist and that they are found in the influence of electrolytes on the osmotic pressure of protein solutions, on the viscosity of certain (but not all) protein solutions, and on the swelling of gels. These peculiarities are as follows:

1. The addition of little acid or alkali to originally isoelectric protein increases the osmotic pressure and viscosity of the protein solution and the swelling of protein gels, until a certain limit is reached; after this the addition of more acid has a depressing effect on these properties.
2. The addition of neutral salts has only a depressing effect on these properties.
3. The depressing effects of electrolytes increase with the valency of that ion which bears a charge opposite to that of the protein ion.
4. Only the valency but not the chemical nature of the crystalloidal ions influences the above-mentioned properties of proteins (except where the electrolyte has secondary effects which influence these properties in an indirect way).

The writer undertook measurements of a new property which had been overlooked in the colloidal literature, namely, the membrane potentials of protein solutions, *i.e.*, the potential differences between solutions of protein salts contained in a collodion bag and outside aqueous solutions free from protein, at the point of osmotic equilibrium. These measurements led to the result that membrane potentials are influenced by electrolytes in a similar way as osmotic pressure, viscosity, or swelling. Since the membrane potentials could be correlated mathematically and quantitatively with Donnan's theory of membrane equilibria, the possibility arose that membrane equilibria might account also for the similar influence of electrolytes on the other three properties; and this was found to be correct.

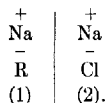
8. DONNAN'S MEMBRANE EQUILIBRIUM

Donnan¹ has shown that when a membrane separates two solutions of electrolytes, one of which contains one ion which cannot diffuse through the membrane while all the other ions can diffuse through the membrane, the result will be an unequal distribution of the diffusible ions on the opposite sides of the membrane. At equilibrium the products of the concentrations of each pair of oppositely charged diffusible ions are the same on the opposite sides of the membrane. This unequal concentration of the crystalloidal ions must give rise to potential differences and osmotic forces, and we intend to show that these forces furnish the explanation of the colloidal behavior of proteins.

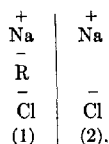
It may be best to quote Donnan's theory in his own words:

We suppose that the membrane (indicated in the following diagram by a vertical line) be impermeable for the anion R of a salt NaR (and also for the non-dissociated part of the salt NaR), but permeable for all the other ions and salts to be considered in this connection . . .

Suppose that in the beginning we have a solution of NaR on one side of the membrane (indicated by a vertical line) and of NaCl on the other side



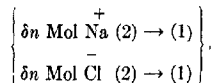
In this case NaCl will diffuse from (2) to (1). In the end the following equilibrium will result:



When this equilibrium is established, the energy required to transport reversibly and isothermally 1 gram molecule Na from (2) to (1) equals the energy which can be gained by the corresponding reversible and isothermal transport of a gram molecule Cl. In other words, we con-

¹ DONNAN, F. G., *Z. Elektrochem.*, vol. 17, p. 572, 1911.

sider the following infinitely small isothermal and reversible change of the system:



The energy which can be gained in this way (*i.e.*, the diminution of free energy) is zero, hence:

$$\delta n \cdot RT \log \frac{[\text{Na}]_2}{[\text{Na}]_1} + \delta n \cdot RT \log \frac{[\text{Cl}]_2}{[\text{Cl}]_1} = 0$$

or

$$[\text{Na}]_2 [\text{Cl}]_2 = [\text{Na}]_1 [\text{Cl}]_1 \quad (1)$$

where the brackets signify molar concentrations.

Wilson¹ has shown that this equation can also be derived electrostatically.

The derivation of the equation need not involve the use of thermodynamics, since it can readily be visualized. In passing from one phase to the other, the oppositely charged ions must move in pairs, since they would otherwise set up powerful electrostatic forces that would prevent their free diffusion. For this reason a sodium or a chlorine ion striking the membrane alone could not pass through it. But, since the membrane is freely permeable to both Na⁺ and Cl⁻, when two oppositely charged ions strike the membrane together, there is nothing to prevent them from passing through into the solution on the opposite side. The rate of transfer of these ions from one solution to the other depends, therefore, upon the frequency with which they chance to strike the membrane in pairs, which is measured by the product of their concentrations. At equilibrium the rate of transfer of Na⁺ and Cl⁻ from Solution II to Solution I exactly equals the rate of transfer of these ions from Solution I to Solution II, from which it follows that the product of the concentrations of these ions has the same value in both solutions.

It is interesting now to note the effect of complicating the system by the introduction of another salt, such as KBr. Following the same line of reasoning, it will be evident that equilibrium will be established only when the product [K⁺] × [Br⁻] has the same value in both solutions, and the same is true for the products [K⁺] × [Cl⁻] and [Na⁺] × [Br⁻]. In fact, with any number of mono-monovalent ionogens present in the system, the product of the concentrations of any pair of diffusible and oppositely charged ions will have the same value in both solutions.

¹ WILSON, J. A., "The Chemistry of Leather Manufacture," New York, 1923.

It can, therefore, be shown thermodynamically as well as electrostatically that equilibrium is reached when the product of the concentrations of a pair of diffusible cations and anions on one side of the membrane is equal to the product of the concentrations of the same pair of diffusible anions and cations on the other side. Since on the side of the non-diffusible (protein) anion the concentration of cations Na is the sum of the cations in combination with the non-diffusible anion plus the cations in combination with the Cl, while on the other side of the membrane the concentration of the Na ions is only that of Na in combination with Cl and equal to the concentration of Cl, it is obvious that Donnan's equation (1) can only be fulfilled if

$$[\text{Na}]_1^+ > [\text{Na}]_2^+$$

and

$$[\text{Cl}]_1^- < [\text{Cl}]_2^-.$$

This inequality of concentration of the diffusible ions on the opposite sides of the membrane accounts, as we shall see, for the influence of electrolytes on all those properties which colloid chemistry has vainly tried to explain on the basis of the dispersion and hydration hypotheses. The reader will notice that the essential condition determining the equilibrium is the existence of two solutions separated by a membrane, one solution containing an ion which cannot diffuse through a membrane which is easily permeable for all the other ions.

This difference in the concentration of the diffusible ions on opposite sides of the membrane must lead to potential differences on opposite sides of the membrane, and Donnan shows that this difference must be (on the basis of Nernst's well-known formula)

$$\pi_1 - \pi_2 = \frac{RT}{F} \log \frac{[\text{Na}]_2^+}{[\text{Na}]_1^+} = \frac{RT}{F} \log \frac{[\text{Cl}]_1^-}{[\text{Cl}]_2^-}$$

or, since $\frac{RT}{F} = 58$ millivolts (at room temperature), the potential

difference on opposite sides of the membrane should be, in millivolts,

$$\pi_1 - \pi_2 = 58 \log \frac{[\text{Na}]_2^+}{[\text{Na}]_1^+} = 58 \log \frac{[\text{Cl}]_1^-}{[\text{Cl}]_2^-}.$$

The writer has tested this consequence of Donnan's theory for solutions of protein salts separated from water by a collodion membrane, with the result that the theory was completely confirmed. Through these measurements of the membrane potentials the correctness of Donnan's theory was proved beyond doubt.

It now becomes clear why the proof for the stoichiometrical character of the reactions of proteins with acids and alkalis is of fundamental importance. Donnan's theory rests on the existence of one kind of non-diffusible ions and this condition is fulfilled by the fact that proteins form ionizable salts with acids and alkalis and that the protein ion cannot diffuse through dialyzing membranes.

It may be pointed out that it is not necessary that the non-diffusible ion be a colloid; it is only necessary that there be a membrane which prevents one type of ion from diffusing; it is immaterial whether or not this latter ion be a crystalloid or a colloid. If we had a membrane impermeable for a SO_4 ion but permeable for Na and Cl ions, solutions of NaCl and Na_2SO_4 separated by the membrane would give rise to the Donnan equilibrium, and the Na_2SO_4 solution would probably resemble a solution of Na proteinate in regard to certain features of colloidal behavior, *e.g.*, osmotic pressure and P.D. against water.

Donnan and his collaborators proved the existence of the inequality of the concentration of the diffusible ions of two salt solutions on the opposite sides of a membrane when one of the ions was not able to diffuse through the membrane. Thus Donnan and Allmand investigated

the distribution of potassium chloride between two compartments separated by a copper ferrocyanide diaphragm, one compartment of which contained potassium ferrocyanide (the membrane being impermeable to the $\text{Fe}(\text{CN})_6$ ion). The higher concentration of potassium chloride on the side free from potassium ferrocyanide, and the relation

of this unequal distribution to the concentration of the chloride and ferrocyanide, were experimentally established. The results obtained agreed, in general, with the view of membrane equilibria proposed by Donnan, but a discussion of the distribution data combined with electromotive-force measurements appeared to show that, at all events in the case of a copper ferrocyanide membrane and potassium ferrocyanide solutions, the phenomena are not so simple as supposed in the theory.¹

More recently Donnan and Garner² investigated the equilibrium concentration of solutions of Na and K ferrocyanides and of Na and Ca ferrocyanides across a copper ferrocyanide membrane, and the results were in general agreement with Donnan's theory. They also investigated a liquid membrane, namely, amyl alcohol, and the electrolytes employed were KCl and LiCl.

So far as the preliminary experiments go, the equilibrium concentration of the Li and Cl ions and the undissociated part of the electrolyte agree with Donnan's theory.

We shall see that Donnan's theory explains the influence of electrolytes on the physical properties of proteins. He foresaw the bearing which his theory was likely to have for colloid chemistry and physiology, as is shown by the following remarks:

In this paper an attempt is made to describe ion equilibria which are bound to occur when certain ions (or their corresponding non-dissociated salt) cannot diffuse through a membrane. Such equilibria possess a great importance for the theory of dialysis and of colloids as well as for the mechanism of the cell and for general physiology.

As far as the writer is aware, Procter and J. A. Wilson were the only authors who attempted the application of Donnan's theory to colloidal problems.

Procter³ proposed in 1914 an ingenious theory of swelling based on Donnan's membrane equilibrium. According to this theory the force which causes the entrance of water into the gel, and thus determines the swelling, is the osmotic pressure of the

¹ DONNAN, F. G. and ALLMAND, A. J., *J. Chem. Soc.*, vol. 105, p. 1963, 1914.

² DONNAN, F. G. and GARNER, W. E., *J. Chem. Soc.*, vol. 115, p. 1313, 1919.

³ PROCTER, H. R., *J. Chem. Soc.*, vol. 105, p. 313, 1914. PROCTER, H. R. and WILSON, J. A., *J. Chem. Soc.*, vol. 109, p. 307, 1916.

excess of crystalloidal ions inside over that outside the gel, this excess being caused by the Donnan equilibrium. The opposing force which limits the swelling is the force of cohesion of the colloidal particles. The later work was done by Procter and Wilson.

According to Procter, the gelatin ions constituting a jelly of gelatin chloride cannot diffuse, and hence can exercise no osmotic pressure, while the chlorine anions in combination with them are retained in the jelly by the electrostatic attraction of the gelatin ions, but exert osmotic pressure. This difference in the diffusibility of the two opposite ions of gelatin chloride gives rise to the establishment of Donnan's membrane equilibrium.

Procter put solid gelatin chloride into an aqueous solution of HCl and determined by titration the distribution of free HCl inside the gel and outside at the time of equilibrium. In this case there exists inside the gel free HCl and gelatin chloride, outside HCl. The relative concentration of free HCl inside and outside at the time of equilibrium is determined by the equation for the Donnan equilibrium

$$x^2 = y(y + z), \quad (1)$$

where x is the concentration of the H and Cl ions in the outside solution, y the concentration of H and Cl ions of the free HCl inside the gel, and z the concentration of Cl ions in combination with the gelatin cation. x and y can be determined experimentally and z can be calculated with the aid of the equation. In other words, the distribution of the H and Cl ions on the opposite sides of a membrane is such that the product of the concentrations of the pair of oppositely charged ions is equal in both phases.

The gelatin salt, like other salts, is highly ionized into the anion and a colloid cation, which either from polymerization or other causes peculiar to the colloid state cannot diffuse and exerts no measurable osmotic pressure, whilst its anion is retained in the jelly by electrochemical attraction of the colloid ion, but exerts osmotic pressure which, on the one hand, causes the mass to swell with absorption of the external solution, and, on the other, expels a portion of the acid, both anion and hydron, from this solution absorbed, the result in equilibrium being that the jelly is poorer in hydron and more concentrated in anion than

the external acid solution, the difference of concentration between anion and hydrion in the jelly being, of course, equal to the ionized anion of the gelatin salt, and electrically balanced by the positive gelatin ions; whilst the hydrion concentration in the jelly is less than that of the outer solution by the amount of acid expelled.¹

By establishing a connection between the volume of the gel and the observed values of x and y , Procter and Wilson were able to calculate the effect of different concentrations of HCl on the swelling of gelatin, and they could show why little acid increased the swelling until a maximum was reached and why the addition of more acid depressed the swelling. They could further show why the addition of neutral salt caused a depression of the swelling.

It is of interest to inquire why this theory of swelling was not accepted and only rarely mentioned in the colloidal literature. In the first place, the application of Donnan's theory to the behavior of proteins requires the proof that proteins form true salts with acids and alkalies and that these salts dissociate electrolytically into a protein ion and a crystalloidal cation or anion. Such an assumption was in conflict with the absorption hypothesis accepted by the colloid chemists. Moreover, the application of the Donnan theory to proteins tacitly implied that only the valency and sign of charge should have an effect on the proteins, while the nature of the ion should have no effect; and this was in conflict with the belief in the Hofmeister ion series. But even authors, like Robertson, who was a champion of the purely chemical conception of the behavior of proteins, refused to accept Procter's theory of swelling.

There should be a measurable potential difference between the gelatin jelly and the external medium. This potential difference has been sought for by Ehrenberg, who was unable to detect any measurable potential between the interior of a jelly and the external medium.²

This gap has been filled by the writer's experiments, which have demonstrated the existence of this potential. The writer

¹ PROCTER, H. R. and WILSON, J. A., *J. Chem. Soc.*, vol. 109, pp. 309-310, 1916.

² ROBERTSON, T. B., "The Physical Chemistry of the Proteins," p. 297, New York, London, Bombay, Calcutta, and Madras, 1918.

has not only been able to furnish support for Procter's theory of swelling but has also been able to show that the potential differences across a membrane separating a solution of a protein salt from pure water fully support Donnan's theory.¹ When a solution of a gelatin-acid salt with monovalent anion, *e.g.*, gelatin chloride (or gelatin phosphate), inside a collodion bag is dipped into pure water, the hydrogen ion concentration as well as the anion concentration on the opposite sides of the membrane are different when osmotic equilibrium is established. The writer was able to show that the potential differences calculated from this difference in the concentration of ions on the basis of Nernst's formula agree with the actually observed P.D., and that the calculated P.D. is the same whether based on a measurement of the difference in the concentration of the hydrogen ions or of the difference in the concentration of the chlorine ions on the opposite sides of the membrane. This latter fact seems a complete proof for the correctness of Donnan's theory of membrane equilibrium, and also a further proof for the correctness of the purely chemical conception of the combination of proteins with acids and alkalies, for unless the proteins form true ionizable salts with acids and alkalies they cannot fulfil the requirements of the Donnan equilibrium.

It was, however, possible to go a step further, inasmuch as these membrane potentials showed the typical colloidal characteristics noticed in connection with viscosity, swelling, and osmotic pressure, namely, the potential difference across the membrane was depressed by the addition of neutral salts, increased by the addition of little acid to isoelectric protein, and depressed by the addition of more acid; the depressing effect was in both cases due to the ion with the opposite sign of charge to that of the protein ion, and, finally, the depressing influence increased rapidly with the valency of the active ion—while the other characteristics of the ion aside from sign and valency had no effect. In this case there was not the slightest doubt that the effects were exclusively the result of the Donnan equilibrium, since they could be mathematically predicted and calculated from the equilibrium formula.

¹ LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 667, 1920-21.

It had been noticed that little acid increases and more acid diminishes the osmotic pressure of a protein solution. This was generally ascribed to an influence of the acid on the degree of dispersion of the protein in solution. The Donnan equilibrium leads to an unequal distribution of the diffusible ions on the opposite sides of the membrane, the total molar concentration of these ions being greater on the side of the protein solution. The writer has been able to show that this difference accounts for the peculiar influence of acid on the osmotic pressure of protein solutions and that if the observed osmotic pressure of such protein solutions is corrected for the unequal distribution of crystalloidal ions on the opposite sides of the membrane on the basis of Donnan's equilibrium equation, it is found that there is practically nothing left for the dispersion theory to explain. The effect of acids and alkalis on osmotic pressure of protein solutions is, therefore, not due to an influence of the acid on the degree of dispersion or hydration or any other so-called colloidal property of the protein, but is the consequence of the excess in the concentration of crystalloidal ions inside the protein solution over the concentration outside. On this basis all the effects of electrolytes on the osmotic pressure of protein solutions can be derived mathematically from Donnan's equilibrium equation and the observations agree within the limits of accuracy of the measurements quantitatively with the values calculated on the basis of this equation.

The fact that the Donnan equilibrium explains the influence of electrolytes on the osmotic pressure of protein solutions furnishes also the key for the understanding of the fact that only the valency, and not the chemical nature of the ions of an electrolyte, influences this property, since the equilibrium equation depends only upon the valency of the ion with which the protein is in combination.

The reason that osmotic pressure, viscosity of protein solutions, and the swelling of protein gels are all influenced in a similar way by electrolytes is that all three properties are in the last analysis functions of one and the same property, namely, osmotic pressure. Procter and Wilson have shown that the swelling of gel under the influence of acid is due to an increase in the osmotic pressure inside the gel. The writer has shown that where the

viscosity of protein solutions is affected by electrolytes in a similar way as is their osmotic pressure, we are dealing with the influence of the electrolytes on the swelling of solid aggregates of protein, and this swelling is due to the osmotic pressure inside the aggregates.¹ It follows from Einstein's theory of viscosity that such a swelling of aggregates must increase the viscosity.

It therefore turns out that two laws of classical chemistry suffice to explain the colloidal behavior of proteins quantitatively and mathematically, and these two laws are the stoichiometrical law and Donnan's theory of membrane equilibria. The proof for this statement is the purpose of this book.

¹ LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 827, 1920-21; vol. 4, pp. 73, 97, 1921-22.

CHAPTER II

QUALITATIVE PROOF OF THE CORRECTNESS OF THE CHEMICAL VIEWPOINT

PREPARATION OF PROTEINS FREE FROM IONOGENIC IMPURITIES

The first problem confronting the chemist is to find a method which permits him to settle definitely the problem whether only one or both ions of a salt combine with a protein. This decision was not possible with the old methods. Those who believe in the adsorption theory assume that both ions of a salt are adsorbed by colloids and Pauli holds that both ions of a salt are adsorbed by the non-ionized molecules of protein.¹

When a block of gelatin is put into a salt solution the solution enters into the interstices between the gelatin molecules constituting the block. When such a block of gelatin is melted, of course, both ions of the salt are found, but nobody can tell whether the salt found was only the salt contained in the interstices of the original gel or whether it was in combination with the gelatin. This difficulty can be circumvented by using solid gelatin in the form of a very fine powder of grains approximately equal in size. When such powdered gelatin is exposed to a salt solution for some time we can ascertain with certainty by a process of washing, whether one or both ions are in combination with the gelatin. After a small mass of the powdered gelatin has been exposed to a salt solution for about 1 hour, it is put on a filter and perfused, with stirring, about six times or more with 25 c.c. of ice-cold distilled water. The water must be cold, since otherwise the granules will coalesce, rendering the process of washing futile. By this procedure it is possible to remove the salt solution between the granules of gelatin, without removing the ions in chemical combination with the gelatin—at least not by the six washings. By using this method of washing we can ascertain whether both or only one of the two oppositely charged ions of a salt enters into combination with gelatin.

¹ PAULI, W., *Fortschritte naturwiss. Forschung*, vol. 4, p. 223, 1912.

Such experiments show that at a given hydrogen ion concentration either the cation or only the anion or neither ion can combine with gelatin; and that it depends solely on the hydrogen ion concentration of the solution which of the three possibilities exists.¹

Proteins are amphoteric electrolytes which exist in three states, according to their hydrogen ion concentration, namely, (1) as non-ionogenic or isoelectric protein; (2) metal proteinate (*e.g.*, Na or Ca proteinate); and (3) protein-acid salts (*e.g.*, protein chloride, protein sulphate, etc.). We will use gelatin as an illustration. At one definite hydrogen ion concentration, namely, that of the isoelectric point, which in the case of gelatin lies at $10^{-4.7}$ N (or in Sørensen's logarithmic symbol at pH = 4.7), gelatin can combine practically with neither anion nor cation of an electrolyte. At a pH > 4.7, gelatin can combine only with cations (forming metal gelatinate, *e.g.*, Na gelatinate); at a pH < 4.7, gelatin combines with anions (forming gelatin chloride, etc.). This was proved in the following way: Doses of 1 gm. of finely powdered commercial gelatin (going through sieve 60 but not through 80), which happened to have a pH of 7.0, were brought to different hydrogen ion concentrations by putting them for 1 hour at about 15°C. into 100 c.c. of HNO₃ solutions varying in concentration from M/8,192 to M/8. Owing to the Donnan equilibrium, the hydrogen ion concentration inside a gelatin granule is lower than that outside. After this, each dose of 1 gm. of gelatin was put on a filter, the acid being allowed to drain off, and each dose was washed once or twice with 25 c.c. of cold water (at 5°C. or less) to remove the greater part of the acid between the granules of the powdered gelatin. These different doses of originally 1 gm. of gelatin, each of which now possessed a different pH, were put for 1 hour each into a separate beaker containing the same concentration, *e.g.*, M/64, of silver nitrate at a temperature of 15°C. Each dose of powdered gelatin was then put on a filter and washed with stirring six or eight times each with 25 c.c. of ice-cold water. This washing serves the purpose of removing the AgNO₃ held in solution between the granules, thus allowing us to ascertain where the Ag is in combination with gelatin and

¹ LOEB, J., *J. Gen. Physiol.*, vol. 1, pp. 39, 237, 1918-19; *Science*, vol. 52, p. 449, 1920; *J. chim. phys.*, vol. 18, p. 283, 1920.

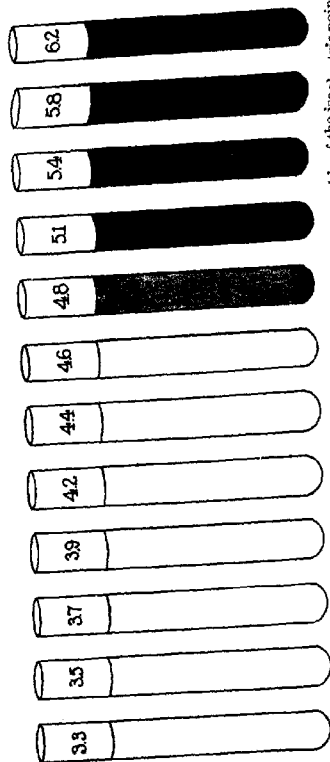


FIG. 1.—Proof that cations combine with proteins only on the alkaline side of the isoelectric point. Powdered gelatin brought to different pH was treated in a dark room with $\pi/64$ AgNO_3 , and then washed with cold water to remove the silver not in combination with gelatin. The gelatin was liquefied, brought to a 1 per cent solution, and the pH was determined. The solutions were then poured into test tubes and exposed to light. In about half an hour the gelatin of pH > 4.7 was dark, while the gelatin of pH 4.7 or less remained permanently clear though exposed to light for over a year. The pH of each gelatin solution is marked at the head of each test tube. (*Facing p. 34*)

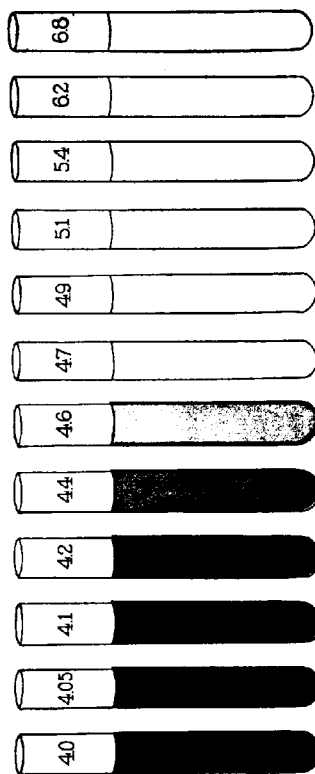


FIG. 2.—Proof that anions combine with proteins only on the acid side of the isoelectric point. Doses of powdered gelatin solutions of different pH were treated with $M/128$ $K_4Fe(CN)_6$ and then washed with cold water. All the samples of gelatin solution of $pH < 4.7$ turned blue (through the formation of some ferric salt), while all the gelatin solutions of pH 4.7 or above remained colorless.

where it is not in combination, since the Ag not in combination with gelatin can be removed by the washing, while the former cannot, or at least only extremely slowly (by altering the pH). After removing the AgNO_3 not in combination with gelatin by washing with cold water, the gelatin is melted by heating to 40°C ., enough distilled water is added to bring the volume of each gelatin solution to 100 c.c., the pH of a sample of each solution is determined potentiometrically, and the solutions are exposed in test tubes to light, the previous manipulations having been carried out in a dark room (with the exception of the determination of pH, for which only part of the gelatin solution was used). In 20 minutes all the gelatin solutions with a $\text{pH} > 4.7$, *i.e.*, from pH 4.8 and above, upon exposure to strong light become opaque and then brown or black, while all the solutions of $\text{pH} < 4.7$, *i.e.*, from 4.6 and below, remain transparent even when exposed to light for months or years (Fig. 1). The solutions of $\text{pH} = 4.7$ become opaque, but remain white, no matter how long they may have been exposed to light. At this pH—the isoelectric point—gelatin is not in combination with Ag, but it is sparingly soluble. Hence, the cation Ag is only in chemical combination with gelatin when the pH is > 4.7 . At pH 4.7 or below, gelatin is not able to combine with Ag ionogenically. This statement was confirmed by volumetric analysis.

The same tests can be made for any other cation, the presence of which can be easily demonstrated. Thus, when powdered gelatin of different pH is treated with NiCl_2 , and the NiCl_2 not in combination with gelatin is removed by washing with cold water, the presence of Ni can be demonstrated in all gelatin solutions with a $\text{pH} > 4.7$ by using dimethylglyoxime as an indicator. All gelatin solutions of pH of 4.8 or above assume a crimson color upon the addition of dimethylglyoxime, while all the others remain colorless. If we use copper instead of Ag or Ni as a cation, treating gelatin with copper acetate, and washing afterwards, the gelatin is blue and opaque when its pH is 4.8 or above, but is colorless and clear for $\text{pH} < 4.7$. Most striking are the results with basic dyes, *e.g.*, basic fuchsin or neutral red, after sufficient washing with cold water; only those gelatin solutions are red whose pH is above 4.7, while the others are colorless.

On the acid side of the isoelectric point, *i.e.*, at $\text{pH} < 4.7$, the gelatin is in combination with the anion of the salt used. This can be demonstrated in the same way by bringing different doses of powdered gelatin to different pH and treating them for 1 hour with a dilute solution of a salt whose anion easily betrays itself, *e.g.*, $\text{M}/128 \text{K}_4\text{Fe}(\text{CN})_6$. If after this treatment the powdered gelatin is washed six times or oftener with cold water to remove the $\text{Fe}(\text{CN})_6$ not in chemical combination with gelatin, and if 1 per cent solutions of these different samples of gelatin are made, it is found that when the pH is < 4.7 the gelatin solution turns blue after a few days (due to the formation of ferric salt), while solutions of gelatin with a pH of 4.7 or above remain permanently colorless (Fig. 2). Hence, gelatin enters into chemical combination with the anion $\text{Fe}(\text{CN})_6$ only when the pH is < 4.7 . The same fact can be demonstrated through the addition of ferric salt when gelatin has been treated with NaCNS , the anion CNS being in combination with gelatin only where the pH is < 4.7 . Acid dyes, like acid fuchsin, combine with gelatin only when the pH is < 4.7 .¹

In this way it can be shown that, when the pH is > 4.7 , gelatin can combine only with cations; when the pH is < 4.7 , gelatin can combine only with anions, while at pH 4.7 (the isoelectric point) gelatin can combine with neither anion nor cation. The idea that both ions are adsorbed or combine with gelatin simultaneously is no longer tenable, since otherwise both ions of the salt should have been discovered on both sides of the isoelectric point.

It follows also that a protein solution is not adequately defined by its concentration of protein but that the hydrogen ion concentration must also be known, since each protein occurs in three different forms—possibly isomers—according to its hydrogen ion concentration.

Let us now return once more to the experiment in which doses of powdered gelatin were brought to a different pH and subse-

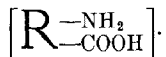
¹ In these experiments it may happen that a few individual granules do not give off their stain at the isoelectric point or on the alkaline side of the isoelectric point, due probably to experimental shortcomings. When the gelatin is melted the solution may show an indication of red. The difference between the gelatin on the alkaline and on the acid side is, however, sufficiently striking even if this slight error interferes.

quently treated for 1 hour with the same concentration of AgNO_3 , *e.g.*, $\text{M}/64 \text{ AgNO}_3$, and then washed. In this case, the exposure to light showed that silver gelatin ate existed only on the alkaline side of the isoelectric point, since only on that side did the gelatin turn black. When we now add enough alkali to the gelatin solutions with a pH of 4.6 or less to bring their pH to 4.8 or above, they will *not* turn black when exposed to light. This shows that the gelatin of pH below 4.6 did not contain any demonstrable quantity of silver.¹ It was conceivable that such gelatin of pH below 4.6 contained Ag in a non-ionogenic form. If this were the case, the fact should have betrayed itself in a blackening upon the addition of enough alkali to bring the pH above 4.7.

When we bring powdered gelatin of $\text{pH} > 4.7$, which has been treated with $\text{M}/64 \text{ AgNO}_3$ and adequately washed, to a pH of 4.7, or below, the silver which was in combination with the gelatin can be removed by washing with cold water, and such gelatin will not turn black when subsequently exposed to light, provided the washing had been adequate.

When we include that part of the gelatin molecule which cannot react with other electrolytes in brackets, while the part of the molecule which is capable of reacting with other electrolytes is kept outside the brackets, we can symbolize our results in the following way:

Isoelectric gelatin is entirely inside the brackets, since at the isoelectric point gelatin can combine neither with anions nor with cations,

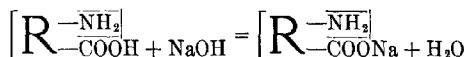


On the alkaline side from the isoelectric point practically only COOH groups of the molecule are capable of reacting with other compounds and we represent the protein molecule on this side in the following form:

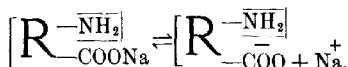
¹ This dogmatic presentation of our results is only approximately correct, since a trace of anion should also combine, theoretically at least, on the alkaline side of the isoelectric point; and a trace of cation on the acid side, at least near the isoelectric point. As a matter of fact, however, this cannot be demonstrated, though the theory of amphoteric electrolytes demands that this should be so.



Such proteins behave as if they were simple (probably polybasic) fatty acids, the rest of the molecule not participating in the reaction. In the presence of a hydroxide, *e.g.*, NaOH, sodium proteinate is formed

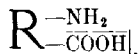


and the sodium proteinate dissociates electrolytically into a protein anion and a Na⁺ ion



When other electrolytes are present, they can, of course, exchange their cation with the Na of the protein salt. Our symbol considers only one COOH group, but it is certain that, as a rule, more than one COOH group of a protein molecule combines with alkali (Bugarszky and Liebermann, Sackur, Robertson, Sørensen, Pauli, Northrop¹).

On the acid side of the isoelectric point only the NH₂ groups of the molecule are capable of reacting with other compounds and we represent the protein molecule on this side in the following form:



In this form the proteins behave like NH₃, which, according to Werner,² is capable of adding an acid, *e.g.*, HCl, the H ion of the acid being added directly to the N while the Cl remains outside

the ring of the 4H in the following way: $\begin{array}{c} \text{H} \\ \text{HNHCl} \\ \text{H} \end{array}$. It has been

shown by G. N. Lewis,³ by W. Kossel,⁴ and by Langmuir⁵ that

¹ NORTHROP, J. H., *J. Gen. Physiol.*, vol. 3, p. 715, 1920-21.

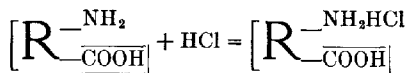
² WERNER, A., "Neuere Anschauungen auf dem Gebiete der anorganischen Chemie," 3d ed., Braunschweig, 1913.

³ LEWIS, G. N., *J. Am. Chem. Soc.*, vol. 38, p. 762, 1916.

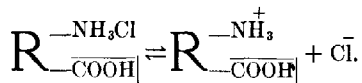
⁴ KOSSEL, W., *Ann. Physik*, vol. 49, p. 229, 1916.

⁵ LANGMUIR, I., *J. Am. Chem. Soc.*, vol. 41, p. 868, 1919.

this idea of Werner is in perfect harmony with the electronic conception of molecular compounds, and we shall give later in this book a direct proof that it holds for proteins. We can therefore say that on the acid side of its isoelectric point the protein particle is able to add acid to its NH_2 groups in the following form:



which dissociates electrolytically into a protein cation and an anion,



While our symbol indicates only one NH_2 group in the molecule, it is certain that more than one NH_2 or NH group is capable of adding an acid molecule.

The simplification in the general chemistry of proteins implied in these experiments is considerable. We only need to remember that on the alkaline side of its isoelectric point the protein behaves as if it were entirely or essentially a fatty acid, only the COOH groups existing in a chemically active form; while on the acid side of its isoelectric point we may again disregard the enormous protein molecule and go on the assumption that the protein consists only of a number of NH_2 groups, each capable of adding the hydrogen ion of an acid.

It is possible, though not proved, that the difference in the behavior of the proteins on the two opposite sides of the isoelectric point is accompanied by an intramolecular change in the protein molecule, and that the protein anion in a metal proteinate may be considered an isomer of the protein cation in protein-acid salt. Such a possibility is suggested by the behavior of indicators, the electrolytic dissociation of which is accompanied by an intramolecular change.

When we mix a metal gelatinate, *e.g.*, sodium gelatinate, with another salt, *e.g.*, MgSO_4 , the Na of the metal gelatinate can be replaced by the Mg of the MgSO_4 , resulting in the formation of magnesium gelatinate. The SO_4 , however, cannot affect the

properties of Na gelatinate, since it cannot (or practically cannot) combine with the gelatin. When, however, we mix gelatin chloride with MgSO_4 , only the SO_4 can affect the properties of the gelatin salt, since the SO_4 can replace the Cl in the gelatin chloride, resulting in the formation of gelatin sulphate. The Mg, however, cannot (or practically cannot) enter into combination with gelatin chloride and hence cannot affect its properties.

When we alter the pH of a gelatin-acid salt, *e.g.*, gelatin chloride, by adding alkali, *e.g.*, NaOH, it will cease to be gelatin chloride as soon as the pH is 4.7, because at this pH the Cl will be given off by the gelatin and the latter will be transformed into the chemically inert isoelectric or non-ionogenic gelatin and into NaCl. The isoelectric gelatin can combine practically neither with anions nor with cations. When we add more NaOH so that the pH is >4.7 , Na gelatinate will be formed. At no time can metal gelatinate (*e.g.*, Na gelatinate) and gelatin-acid salt (*e.g.*, gelatin chloride) exist simultaneously (except in traces, which in the case of gelatin are below the limits of analytical demonstration). When we have Na gelatinate and add acid, *e.g.*, HCl, the gelatin salt will give off its Na and become isoelectric gelatin as soon as the pH = 4.7. This isoelectric gelatin is chemically inert, being practically unable to combine with either anion or cation. When we add more HCl, gelatin chloride will be formed.

These experiments show that proteins behave like amphoteric electrolytes, forming definite salts with acids or bases, but that they cannot practically combine simultaneously with the cation and the anion of a neutral salt. The idea of the existence of adsorption compounds between non-ionized molecules of proteins and molecules of neutral salts is not in harmony with these experiments.

In 1918 the writer¹ published a simple method of preparing ash-free proteins, based on the fact that at the isoelectric point proteins or, perhaps, more correctly, proteins free from ionogenic impurities can combine neither with anions nor with cations. Hence, if we wish to prepare gelatin or casein free from ionogenic impurities, we must bring these proteins in powdered form to the isoelectric point and wash them. This is of importance for all industries using proteins, as well as for scientific work. In the

¹ LOEB, J., *J. Gen. Physiol.*, vol. 1, p. 237, 1918-19.

writer's work isoelectric protein was always used as the starting point for experiments.

The procedure for preparing isoelectric protein is simple enough. It is only necessary to determine the pH of a given protein solution potentiometrically, and then to add very gradually as much acid or alkali as is required to bring it to the isoelectric point.

The following method was used to prepare larger quantities of approximately isoelectric gelatin: 50 gm. of commercial powdered Cooper's gelatin, which happened to have a pH of 6.0 to 7.0, were put into 3,000 c.c. of $M/128$ acetic acid in a jar at 10°C ., and stirred frequently. After 30 minutes the supernatant liquid was decanted and fresh $M/128$ acetic acid at 10°C . was added to equal the original volume. The mass was frequently stirred, and after 30 minutes the acid was again decanted and replaced by an equal volume of distilled water at 5°C . The gelatin was well stirred and then filtered by suction through towel cloth in a Buchner funnel. It was then washed in the funnels five times each with 1,000 c.c. of H_2O at 5°C . After all the water was drained off the gelatin was transferred from the Buchner funnel into a large beaker which was then heated in a water bath to about 50°C . till the gelatin was melted. The concentration of the gelatin was determined by evaporating to dryness, using 10 c.c. of the melted gelatin in an electric oven at 90 to 100°C . for 24 hours.

One hundred cubic centimeters of a 1 per cent gelatin solution prepared in this way had no more than 1 mgm. of ash—apparently $\text{Ca}_3(\text{PO}_4)_2$, *i.e.*, the salt contained in the solution was $M/30,000$. Salt in this concentration does not affect the physical properties of proteins, such as osmotic pressure, viscosity, P.D., swelling, or precipitability, as will be shown in this volume. The following is a result of an ash determination made by Dr. D. I. Hitchcock on a sample of gelatin selected at random. The stock solution contained 12.69 per cent gelatin.

	SAMPLE No. 1	SAMPLE No. 2
Volume of solution.....	20 c.c.	10 c.c.
Weight of dry gelatin.....	2.535 gm.	1.269 gm.
Weight of ash.....	0.0024 gm.	0.0012 gm.
Obtained qualitative tests for Fe^{+++} , Ca^{++} , and PO_4^{\equiv} , negative tests for Cl^- and SO_4^{\equiv} .		

Miss Field¹ has shown that by carrying the washing process a step further the last traces of ash can be removed from the powdered gelatin. In bringing powdered gelatin to the isoelectric point and washing with water of the pH of the isoelectric point we can quickly make the gelatin completely ash-free. If the protein is soluble at this point (as is the case with crystalline egg albumin), it is only necessary to carry out the dialysis at the pH of the isoelectric point to obtain the protein free from ionogenic impurities.²

This fact is a further support of our contention that at the *isoelectric point proteins can combine with neither anion nor cation*.

We may call attention to one interesting fact which is in harmony with these results. It has always been known that pepsin digestion occurs in nature in an acid medium. The reason for this connection of an acid reaction with pepsin digestion was cleared up by Northrop,³ who found that the hydrogen ion concentration at which pepsin commences to act on a protein varies with the isoelectric point of the protein and that the action always occurs on the acid side of the isoelectric point. It seems to follow from the experiments of Pekelharing and Ringer⁴ that pepsin is an anion like Cl which can only combine with a positive protein ion. This combination between pepsin and positive protein ion seems to be the prerequisite for the falling apart (or digestion) of the protein ion.

It was known that trypsin digestion occurs generally in an alkaline medium. Northrop has shown that the hydrogen ion concentration at which trypsin commences to act on a protein varies also with the isoelectric point of the protein and that the digestive action of trypsin occurs on the alkaline side of the isoelectric point.⁵

¹ FIELD, A. M., *J. Am. Chem. Soc.*, vol. 43, p. 667, 1921.

² Miss Field's paper as well as the writer's paper referred to were overlooked by C. R. SMITH (*J. Am. Chem. Soc.*, vol. 43, p. 1350, 1921), who also describes a method of preparing ash-free gelatin.

³ NORTHROP, J. H., *J. Gen. Physiol.*, vol. 3, p. 211, 1920-21.

⁴ PEKELHARING, C. A. and RINGER, W. E., *Z. physiol. Chem.*, vol. 75, p. 282, 1911.

⁵ NORTHROP, J. H., *J. Gen. Physiol.*, vol. 5, p. 263, 1922-23.

The qualitative color tests described in this chapter have thus far been carried out only with gelatin. It is quite possible that in the case of other proteins the point of color change may turn out not to be so sharp as in the case of powdered gelatin. The isoelectric point is that point where the ionization of an amphoteric electrolyte is a minimum, but it need not be zero in all cases.

CHAPTER III

METHODS OF DETERMINING THE ISOELECTRIC POINT OF PROTEIN SOLUTIONS

The results of the preceding chapter make it clear that whenever work with amphoteric electrolytes is contemplated it becomes necessary to ascertain first the isoelectric point of the substance; at the isoelectric point the material can be most easily freed from ionogenic impurities. There can be no doubt that many of the substances exhibiting colloidal behavior are amphoteric electrolytes.

Hardy and Michaelis determined the isoelectric point by observations on the migration of particles in the electrical field. Other methods are available for this purpose, some of which are often more convenient than Hardy's original method. These methods are based on the fact that at the isoelectric point the osmotic pressure, the viscosity, the amount of alcohol required for precipitation (Fenn's¹ so-called alcohol number), the conductivity, the swelling, and the P.D. are all a minimum. When the curves representing the values of these properties are plotted as ordinates over the pH as abscissæ, the curves show a sharp drop at the isoelectric point. If, therefore, a protein is brought to different pH by adding acid or alkali, and if any of the properties mentioned is determined, the approximate position of the isoelectric point can be inferred from the minimum point of the property which is used as a test. The writer has found it most convenient to use osmotic pressure experiments in the case of proteins.

The following older experiment by the writer may serve as an illustration.² A number of doses, each containing 1 gm. of finely powdered Cooper's gelatin which had a pH of a little over 7.0

¹ FENN, W. O., *J. Biol. Chem.*, vol. 33, pp. 279, 439; vol. 34, pp. 141, 415, 1918.

² LOEB, J., *J. Gen. Physiol.*, vol. 1, p. 363, 1918-19.

and consisted partly of Ca gelatinates, were put for 30 minutes at 15°C. into beakers containing 100 c.c. of HBr of different concentrations, varying from M/8 to M/8,192; and, as a control, 1 gm. of gelatin was put for 30 minutes at 15°C. into 100 c.c. of distilled water. Each dose was then put into a cylindrical funnel and the acid allowed to drain off. The powdered gelatin in the funnel was then perfused six or eight times, with constant stirring, each time with 25 c.c. of cold water—*i.e.*, water not above 5°C.—to remove the excess of acid and the salts. The water must be cold to prevent the powdered granules from coalescing, since otherwise the washing would be incomplete. After the liquid was drained off from the filter, the volume (*i.e.*, the relative swelling of the gelatin) was measured; then the gelatin was melted by heating to 45°C. and enough water was added to bring the volume in each case to 100 c.c. Then the conductivity, osmotic pressure, and viscosity were measured in a way to be described in a later chapter, and the pH was also determined, either colorimetrically (which gives fairly accurate results with gelatin but not with the other proteins) or preferably with the hydrogen electrode. In the experiment represented in Fig. 3 the pH was measured colorimetrically. A glance at the figure shows that the ordinates of the curves representing the values for osmotic pressure, conductivity, swelling, etc. drop very sharply at pH 4.7, *i.e.*, the isoelectric point of gelatin. By this method the approximate location of the isoelectric point can be recognized at a glance from the osmotic pressure measurements, the conductivity measurements, etc.

The lowest curve in Fig. 3 represents titration for Br. Gelatin should exist in the form of gelatin bromide only on the acid side of the isoelectric point and titration for Br should be negative when the pH is above 4.7. The curve shows that no Br was found when pH was equal or greater than 4.7, while it was found on the acid side increasing in quantity the lower the pH. On the alkaline side of the isoelectric point the gelatin existed still in the state of Ca gelatinates. In this experiment the mass of the gelatin was diminished by solution and washing to 0.8 gm. or possibly a little less.

We shall see later that when powdered gelatin is put into an acid solution, *e.g.*, N/100 or N/1,000 HBr, the concentration of

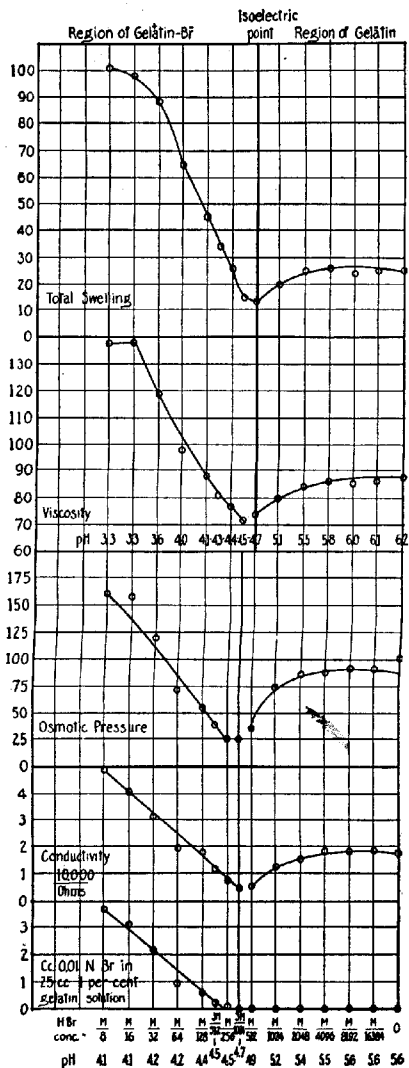


FIG. 3.—Showing that the physical properties of gelatin are a minimum at the isoelectric point.

the acid inside the gelatin granules is considerably lower than in the outside solution. This is due to the establishment of a Donnan equilibrium.

All properties of proteins which increase with ionization must have a minimum at the isoelectric point, since at this point the degree of ionization is a minimum. This is also true for the amino-acids, which are pure crystalloids. While the colloidal behavior of proteins depends on their ionization, ionization in itself does not necessarily give rise to the colloidal behavior of proteins. This is only the case under certain conditions, namely, when the protein ions are prevented from diffusing, while small ions like H or Cl can diffuse. Colloidal properties of proteins, like swelling or osmotic pressure, show also a minimum of their values at the isoelectric point, because the ionization of proteins is a minimum at that point; but it would be an error to infer that only colloidal properties of proteins show a minimum at the isoelectric point.

CHAPTER IV

QUANTITATIVE PROOF OF THE CORRECTNESS OF THE CHEMICAL VIEWPOINT

1. THE NATURE OF THE COMPOUND BETWEEN ISOELECTRIC PROTEIN AND ACID

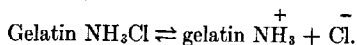
The qualitative experiments of the second chapter did not permit us to decide whether ions combine with proteins stoichiometrically (*i.e.*, by the purely chemical forces of primary valency), or according to the empirical rule of adsorption, as is assumed in colloid chemistry. A decision can be rendered, first, by an investigation of the nature of the compound between acids and isoelectric protein, and second, by titration and combination curves.¹ We will first take up the study of the nature of protein-acid salts.

Our solutions contain generally 1 gm. of isoelectric protein in 100 c.c., and such solutions will be called 1 per cent protein solutions. When 1 per cent solutions of albumin sulphate or 1 per cent solutions of gelatin chloride are mentioned, this means that 1 gm. of originally isoelectric albumin or gelatin was in 100 c.c. of the solution. The concentration of the stock solution of isoelectric gelatin, albumin, or casein was determined by measuring the dry weight of the solution.

When different quantities of 0.1 *N* acid, *e.g.*, HCl, are added to the same quantity of protein, *e.g.*, 1 gm. of isoelectric gelatin or crystalline egg albumin, bringing the volume of the solution always to 100 c.c., it is found that the resulting hydrogen ion concentration of the solution is different from the pH which is found when the same amount of acid is added to the same quantity of pure water. This is due to the fact that part of the acid combines with the protein, as originally suggested by Bugarszky

¹ LOEB, J., *J. Gen. Physiol.*, vol. 1, p. 559, 1918-19; vol. 3, p. 85, 1920-21.

and Liebermann.¹ According to Werner's² idea, the HCl should combine with the NH₂ groups of the protein molecule in the same way as if HCl were added to NH₃, thus forming a salt of the type RNH₃Cl. This is intelligible on the basis of the recent theories of G. N. Lewis³, Kossel,⁴ and Langmuir.⁵ Gelatin chloride may, therefore, be expected to dissociate electrolytically in the following way:



Hence the concentration of the free Cl ions in an aqueous solution of HCl should remain the same if a small amount of isoelectric gelatin is added, provided the electrolytic dissociation is complete. This was tested by comparing the pCl of HCl solutions with and without gelatin (Table I). Both the pH and the pCl were measured electrometrically. For the measurements of the hydrogen ion concentration, the hydrogen electrode was used; for the measurements of the chlorine ion concentration, calomel electrodes were used.

Table I gives the result. The first column of the table gives the cubic centimeters of 0.1 N HCl in 100 c.c. of solution. The second column gives the pH; and the third the pCl of the aqueous HCl solution free from gelatin. As was to be expected, the pH and pCl are identical within the limits of accuracy of the measurements. The fourth and fifth columns give the pH and pCl when the acid is contained in 100 c.c. of a 1 per cent solution of originally isoelectric gelatin instead of in water without gelatin. By comparing column 5 with column 3 in Table I, the reader will notice that the pCl is the same in the presence and in the absence of originally isoelectric gelatin. A comparison of the second and fourth columns of Table I shows that the pH is less in the solution free from gelatin than in the solution with gelatin.

¹ BUGARSZKY, S. and LIEBERMANN, L., *Arch. ges. Physiol.*, vol. 72, p. 51, 1898.

² WERNER, A., "Neuere Anschauungen auf dem Gebiete der anorganischen Chemie," 3d ed., Braunschweig, 1913.

³ LEWIS, G. N., *J. Am. Chem. Soc.*, vol. 38, p. 762, 1916.

⁴ KOSSEL, W., *Ann. Physik*, vol. 49, p. 229, 1916.

⁵ LANGMUIR, I., *J. Am. Chem. Soc.*, vol. 41, p. 868, 1919; vol. 42, p. 274, 1920.

TABLE I

Cubic centimeters of 0.1 N HCl in a 100-c.c. solution	Solution containing no gelatin		Solution containing 1 gm. of isoelectric gelatin in 100 c.c.	
	pH	pCl	pH	pCl
2	2.72	2.72	4.2	2.68
3	2.52	2.54	4.0	2.53
4	2.41	2.39		
5	2.31	2.29	3.60	2.33
6	2.24	2.26	3.41	2.25
7	2.16	2.18	3.23	2.18
8	2.11	2.12	3.07	2.11
10	2.01	2.01	2.78	2.025
15	1.85	1.85	2.30	1.845
20	1.72	1.76	2.06	1.76
30	1.55	1.59	1.78	1.60
40	1.43	1.47	1.61	1.47

These facts prove that HCl combines with gelatin in the way Werner's theory suggests, namely, part of the H ions of the HCl becoming part of a complex gelatin cation of the type gelatin-NH_3^+ , while the Cl ions are not affected by the presence of the protein. The addition of HCl to isoelectric gelatin has, therefore, a similar effect to that of the addition of free HCl to NH_3 ; in the one case gelatin chloride is formed, in the other case ammonium chloride. There is, however, this difference between the two cases, that, while ammonium chloride is only slightly hydrolyzed, gelatin chloride is hydrolyzed to a considerable extent. This hydrolysis is of importance, since it leads to the conclusion that there must always be a definite equilibrium between free acid, the dissociated salt, gelatin chloride, and non-ionized isoelectric gelatin.

It was found that whenever the same amount of acid was added to the same amount, *e.g.*, 1 gm., of originally isoelectric gelatin, making up the volume to 100 c.c. by adding water, the pH of the solution was always the same; so that we can say

how much Cl is in combination with the protein if we know the pH of the gelatin chloride solution and the concentration of originally isoelectric gelatin. The lower the pH the more chloride enters into combination with the protein, until finally all the protein is transformed into protein chloride.

Similar experiments were made by Hitchcock for the combination of HCl with other proteins, namely, crystalline egg albumin, edestin, casein, and serum globulin. The results were similar to those with gelatin.¹

When alkali is added to isoelectric gelatin, an equilibrium is established between metal proteinate, non-ionized protein, and free alkali (above pH 4.7). Similar results had been obtained by Sørensen.²

These experiments then show that acids combine with protein in a way similar to that in which they combine with crystalline bases, e.g., NH_3 or amino-acids. Manabe and Matula,³ as well as Pauli,⁴ had also made such experiments with gelatin and blood albumin which to some extent agree with our results but from which they drew conclusions concerning their electrolytic dissociation with which we cannot quite agree.

2. THE TITRATION CURVES OF GENUINE PROTEINS WITH ACIDS

It can be shown by titration experiments that acids and bases combine with proteins in the same way as they combine with crystalline compounds, namely, by the purely chemical forces of primary valency. It is known that a weak dibasic or tribasic acid gives off one hydrogen ion more readily than both or all three, and that it depends on the hydrogen ion concentration of the solution whether one or two or three H ions are dissociated from a tribasic acid. Thus H_3PO_4 will give off only one H ion as long as the pH is below 4.6. Oxalic acid, which is a stronger acid, will act like a monobasic acid below a pH of about 3.0,⁵

¹ HITCHCOCK, D. I., *J. Gen. Physiol.*, vol. 5, p. 383, 1922-23.

² SØRENSEN, S. P. L., "Studies on Proteins," *Compt.-rend. trav. lab. Carlsberg*, vol. 12, Copenhagen, 1915-17.

³ Manabe, K. and Matula, J., *Biochem. Z.*, vol. 52, p. 369, 1913.

⁴ Pauli, W., "Kolloidchemie der Eiweisskörper," Dresden and Leipsic, 1920.

⁵ HILDEBRAND, J. H., *J. Am. Chem. Soc.*, vol. 35, p. 847, 1913. MICHAELIS, L., "Die Wasserstoffionenkonzentration," Berlin, 1914. CLARK, W. M., "The Determination of Hydrogen Ions," Baltimore, 1920.

while above this pH it acts more and more like a dibasic acid. In a strong dibasic acid, like H_2SO_4 , both H ions are held with so small an electrostatic force that even at a pH of 3.0 or considerably below the acid acts as a dibasic acid. If the forces which determine the reaction between these acids and proteins are purely chemical, it would follow that three times as many cubic centimeters of 0.1 N H_3PO_4 should be required to bring 100 c.c. of 1 per cent solution of isoelectric gelatin to a given pH below 4.6, e.g., 3.0, as are required in the case of HNO_3 or of HCl; while it should require just as many cubic centimeters of 0.1 N H_2SO_4 as of 0.1 N HCl. Twice as many cubic centimeters of 0.1 N oxalic acid should be required to bring isoelectric gelatin to a pH of 3.0 or below as are required in the case of HCl. It can be shown that these predictions are true.¹ In these experiments carefully prepared isoelectric proteins, as free from ionogenic impurities as is possible, must be used.

Crystalline egg albumin was prepared according to Sørensen's method,² and crystallized three times. The only difference in procedure was in the dialysis. Instead of putting the water under negative pressure, as was done by Sørensen, pressure was put on the egg albumin by attaching a long glass tube full of water to the dialyzing bag so that the solution was under about 150 cm. water pressure during dialysis. This was necessary to avoid too great an increase in volume. The same stock solution of albumin served for all the experiments and was diluted before the experiment to a 1 per cent solution. The concentration of ammonium sulphate left in the solution was between M/1,000 and M/2,000. The pH of the stock solution was about 5.20. By adding about 1 c.c. 0.1 N HCl to 100 c.c. of a 1 per cent solution of this albumin the solution was brought to the isoelectric point of the egg albumin, which is, according to Sørensen, at pH = 4.8.

The 1 per cent solutions were made up with different quantities of acid (or alkali) and the pH of the albumin solution was determined electrometrically. In Fig. 4 are plotted the titration curves, in which the pH are the abscissæ while the ordinates are

¹ The experiments to be described are from LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 85, 1920-21.

² SØRENSEN, S. P. L., "Studies on Proteins," *Compt.-rend. trav. lab. Carlsberg*, vol. 12, Copenhagen, 1915-17.

the cubic centimeters of 0.1 N acid required to bring the 1 per cent solutions of originally isoelectric crystalline egg albumin to different pH. The curves represent these titration values for

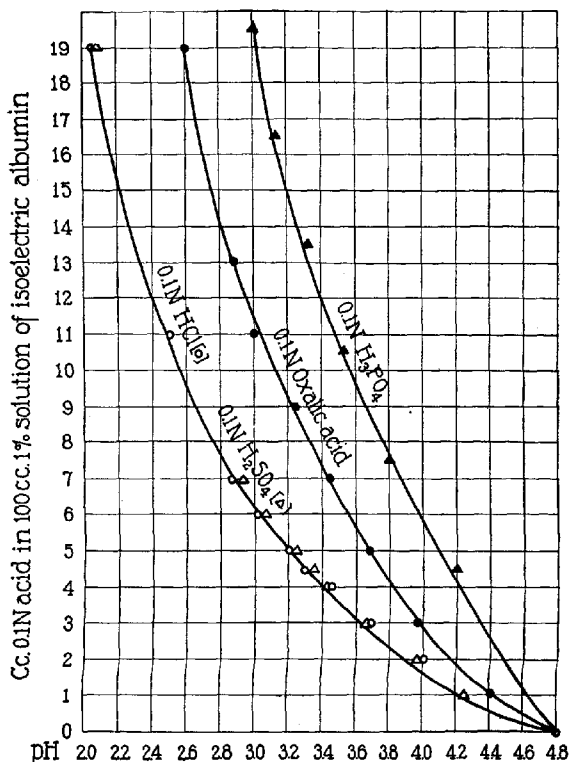


FIG. 4.—The ordinates represent the number of cubic centimeters of 0.1 N HCl, H₂SO₄, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric crystalline egg albumin to the pH indicated on the axis of abscissae. Enough H₂O was added to bring the albumin and acid to a volume of 100 c.c. For the same pH, the ordinates for HCl, H₂SO₄, and phosphoric acid are approximately 1:1:3. The ratio of HCl to oxalic acid is a little less than 1:2 when pH is > 3.0.

four acids, HCl, H₂SO₄, H₃PO₄, and oxalic acid. Beginning with the lowest curve, we notice that the curve is the same for 0.1

N HCl and 0.1 N H_2SO_4 , since both are strong acids; or, in other words, H_2SO_4 combines in equivalent proportions with egg albumin. The curve for H_3PO_4 is the highest curve, and if we compare the values for H_3PO_4 with those of HCl (or H_2SO_4) we notice

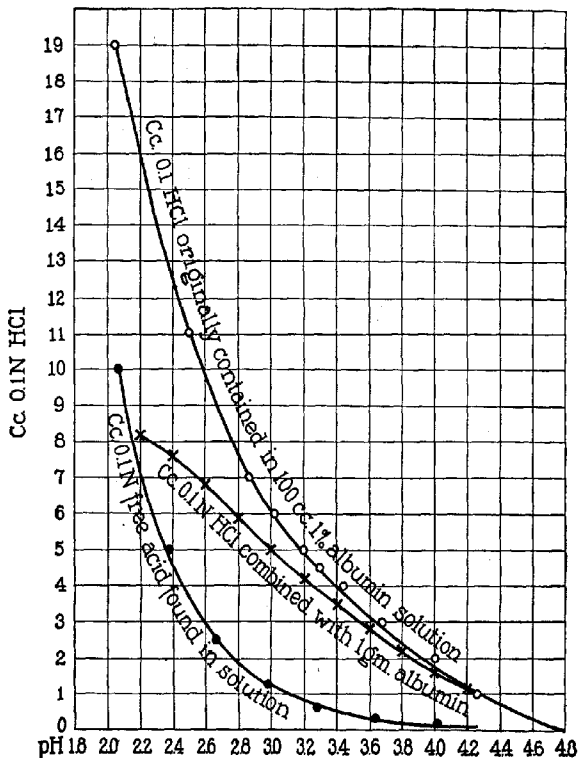


FIG. 5.—Method of determining the amount of acid in combination with 1 gm. of albumin from titration curve and pH curve.

that for each pH the ordinate for H_3PO_4 is as nearly three times as high as that for HCl as the accuracy of our experiments permits. This means that phosphoric acid combines with albu-

min (inside of the range of pH of our experiment) in molecular proportions and that the anion of albumin phosphate is the monovalent anion H_2PO_4 .

The values for oxalic acid are for pH below 3.2 almost, but not quite, twice as high as those for HCl, indicating that for these values of pH oxalic acid combines to a greater extent in molecular and only to a small extent in equivalent proportions with albumin.

That the combining ratios of the four acids named with crystalline egg albumin are the same as those which would be found if we substituted the crystalloidal base NH_3 for the colloid egg albumin, titrating in the same range of pH, can be shown in the following way:

From the titration curves just discussed the amount of acid in combination with 1 gm. of originally isoelectric crystalline egg albumin in a 1 per cent solution of this protein at different pH can easily be calculated. Let us assume the acid added to isoelectric albumin to be HCl. If, *e.g.*, at pH 3.0, 6 c.c. of 0.1 N HCl are contained in 100 c.c. of the 1 per cent solution of the originally isoelectric albumin (as indicated in Fig. 4), part of the acid is in combination with the albumin and part is free. How much is free is known from the pH of the albumin chloride solution, namely, 1 c.c., since in the example selected the pH is 3.0 (Fig. 4). If 1 c.c. is deducted from 6 c.c., it is found that at pH 3.0, 5 c.c. of 0.1 N HCl are in combination with 1 gm. of originally isoelectric crystalline egg albumin in a 100-c.c. solution (Fig. 5). A curve is constructed in which the abscissæ are the pH while the ordinates are the cubic centimeters of 0.1 N HCl contained in 100 c.c. of an aqueous solution, *without protein*. The pH of these aqueous solutions without albumin was also determined with the hydrogen electrode. If the ordinates of this latter curve are deducted from the ordinates of the titration curve in Fig. 4 containing 1 per cent of originally isoelectric albumin chloride, we get a curve whose ordinates give the number of cubic centimeters of 0.1 N HCl in actual combination with 1 gm. of originally isoelectric albumin in a 100-c.c. solution (middle curve Fig. 5).

Figure 6 contains the combination curves whose ordinates give the amount of cubic centimeters of 0.1 N HCl, H_2SO_4 , $\text{H}_2\text{C}_2\text{O}_4$, and H_3PO_4 in combination with 1 gm. of originally isoelectric egg albumin at different pH. It appears again that the combination

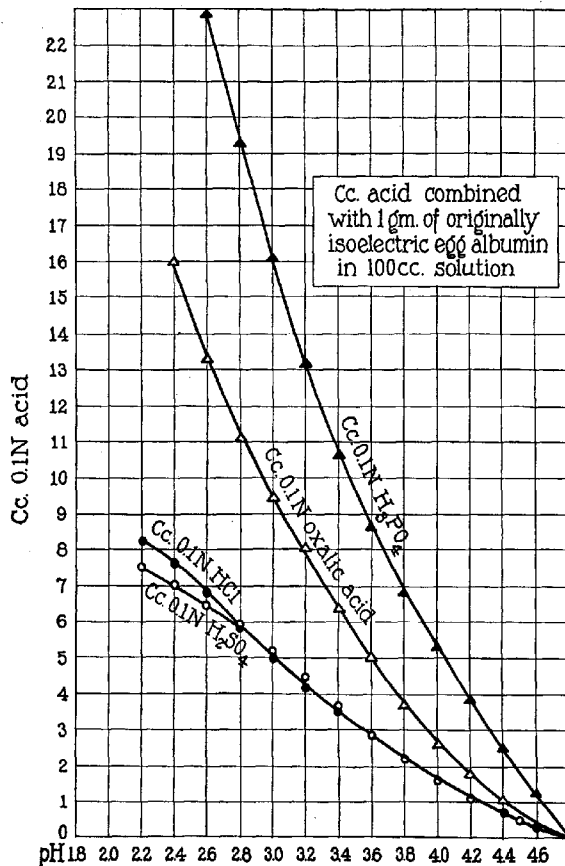


FIG. 6.—Proof of the stoichiometrical character of the combination of acids with isoelectric albumin. The same mass of albumin combines with three times as many cubic centimeters of 0.1 N H_3PO_4 as with HCl or H_2SO_4 ; and with twice as many cubic centimeters of 0.1 N oxalic acid below pH 3.0.

curves for HCl and H_2SO_4 practically coincide, as the purely chemical theory demands, that the oxalic acid curve is higher, and that the phosphoric acid curve is still higher. What is of greater importance is that for the same pH the ordinates of the H_3PO_4 curve are always approximately three times as high as the ordinates of the curves for HCl and H_2SO_4 .

The results in Table II show the actual numbers of cubic centimeters of each of the four acids in combination with 1 gm. of originally isoelectric crystalline egg albumin in a 100-c.c. solution. The values for HCl and H_2SO_4 are identical. Those for H_3PO_4 are within the limits of accuracy always three times as high as those for HCl. Thus, at pH 4.0, 1.7 c.c. of 0.1 N HCl or H_2SO_4 are combined with 1 gm. of albumin, while 5.3 c.c. of 0.1 N H_3PO_4 are in combination; at 3.4, 3.5 c.c. of 0.1 N HCl or H_2SO_4 and 10.6 c.c. of 0.1 N H_3PO_4 .

In the case of oxalic acid, we notice that at pH above 3.6 the number of cubic centimeters of 0.1 N oxalic acid in combination with 1 gm. of albumin is less than twice that of HCl and that the difference is the greater the higher the pH. At pH = 3.2 and below, practically twice as many cubic centimeters of oxalic acid are at the same pH in combination with 1 gm. of originally isoelectric albumin as are of HCl. Thus, at pH 2.6, 6.7 c.c. of 0.1 N HCl and 13.3 c.c. of 0.1 N oxalic acid are in combination with 1 gm. of albumin; at pH 3.0, 5.0 c.c. of 0.1 N HCl and 9.5 c.c. of 0.1 N oxalic acid. These figures correspond to the results to be expected on the basis of Hildebrand's titration experiments against inorganic bases.

These quantitative results prove that when acid is added to a given quantity of originally isoelectric albumin, at the same pH of the solution equal quantities of hydrogen ions enter into combination with the protein, regardless of whether a strong acid (*e.g.*, HCl or H_2SO_4) or a weak acid (*e.g.*, H_3PO_4) is added. This fact shows that proteins combine stoichiometrically with acids. The simplest assumption is that the hydrogen ions combine chemically with the protein. That these simple facts had not been discovered earlier is the consequence of the failure of the workers to consider the hydrogen ion concentration of their solutions. Had this been done, nobody would have thought of

TABLE II.—CUBIC CENTIMETERS OF 0.1 N ACID IN COMBINATION WITH 1 GM. OF ORIGINALLY ISOELECTRIC CRYSTALLINE EGG ALBUMIN IN A 100-C.C. SOLUTION

pH	HCl, cubic centimeters	H ₂ SO ₄ , cubic centimeters	Oxalic acid, cubic centimeters	H ₃ PO ₄ , cubic centimeters
4.2	1.15	1.15	1.8	3.8
4.0	1.7	1.7	2.6	5.3
3.8	2.3	2.3	3.7	6.8
3.6	2.9	2.9	5.0	8.6
3.4	3.5	3.5	6.3	10.6
3.2	4.2	4.3	8.0	13.1
3.0	5.0	5.1	9.5	16.1
2.8	5.8	5.9	11.1	19.3
2.6	6.7	6.5	13.3	22.9
2.4	7.6	7.0	16.0	

suggesting that acids combine with proteins according to the adsorption formula.

These titration experiments are of especial value because crystalline egg albumin is for the present probably the purest protein available.

The same proof can be furnished in the case of other proteins, *e.g.*, gelatin. A stock solution of isoelectric gelatin was used for the experiment. The isoelectric gelatin was prepared by putting the powdered gelatin of pH 7.0 into *m*/128 acetic acid (100 c.c. of *m*/128 acid for 1 gm. of gelatin) for 1 hour at 15°C., and then washing four or five times with cold water (5°C). An 8 per cent stock solution was prepared; the concentration of the gelatin was ascertained by a determination of the dry weight. To 50 c.c. of a 2 per cent solution of isoelectric gelatin were added different quantities of acid and the volume was made up to 100 c.c. by adding enough H₂O, usually of a pH of about 5.6. It was ascertained how many cubic centimeters of 0.1 *N* different acids were required to bring 1 gm. of isoelectric gelatin in a 1 per cent solution to the same pH.

In Fig. 7 the abscissæ are the pH, while the ordinates are the number of cubic centimeters of 0.1 *N* HCl, H₂SO₄, H₂ oxalate,

and H_3PO_4 contained in a 100-c.c. solution of originally isoelectric gelatin to the same pH.

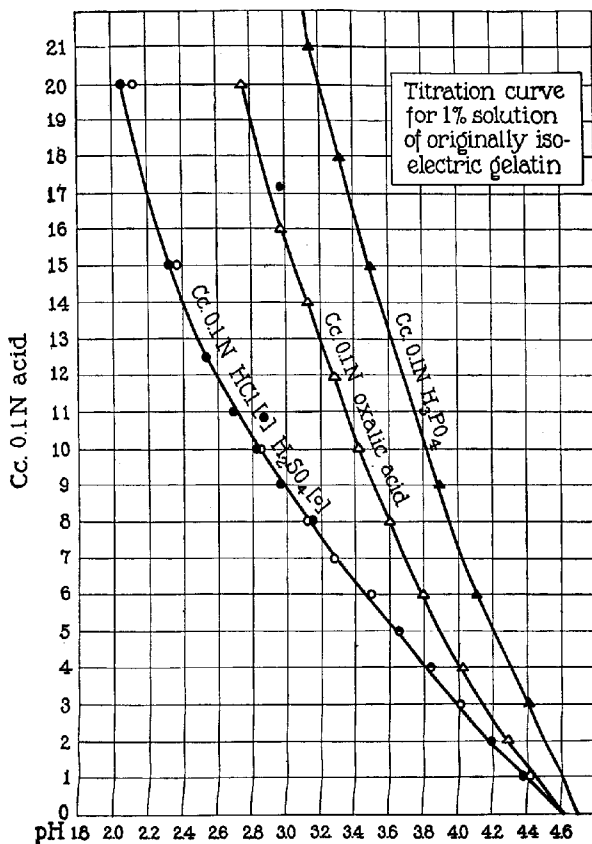


FIG. 7.—Titration curves for 1 per cent solutions of originally isoelectric gelatin, proving the stoichiometrical character of combination of acids with gelatin (see legend under Fig. 4).

It is again obvious that the curves for HCl and H_2SO_4 are practically identical, while the ordinates of the curve for H_3PO_4

are approximately three times and those for oxalic acid about twice as high as those for HCl or H_2SO_4 for the same pH, as long

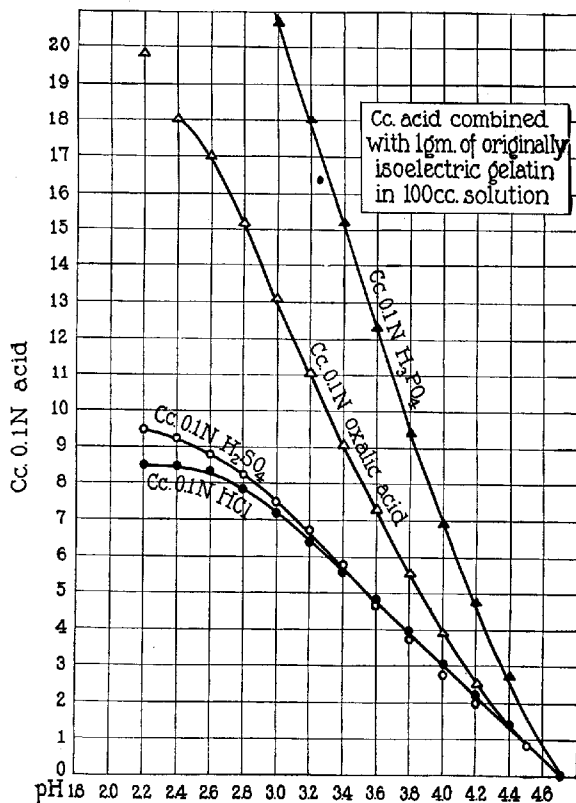


FIG. 8.—Combination curves of acids with gelatin, confirming the stoichiometrical character of the combination.

as the pH is below 3.2, while above 3.2 the curve for oxalic acid deviates the more from that ratio the higher the pH, as the theory demands.

The curves in Fig. 8 represent the values for the cubic centimeters of 0.1 *N* acid found in combination with 1 gm. of originally isoelectric gelatin in a 100-c.c. solution at different pH. The results are tabulated in Table III. The table shows that within the limits of accuracy of the experiments, at the same pH, approximately equal numbers of cubic centimeters of 0.1 *N* HCl and 0.1 *N* H₂SO₄ are in combination with 1 gm. of originally isoelectric gelatin in a 100-c.c. solution, while about three times as many cubic centimeters of 0.1 *N* H₃PO₄ are in combination. The number of cubic centimeters of 0.1 *N* oxalic acid in combination with 1 gm. of gelatin is less than twice that of HCl as long as the pH is above 3.0, while below 3.0 the combining ratio of the two acids is approximately as 1:2, as the theory demands.

TABLE III.—CUBIC CENTIMETERS OF 0.1 *N* ACID IN COMBINATION WITH 1 GM. OF ORIGINALLY ISOELECTRIC GELATIN IN A 100-C.C. SOLUTION

pH	HCl, cubic centimeters	H ₂ SO ₄ , cubic centimeters	Oxalic acid, cubic centimeters	H ₃ PO ₄ , cubic centimeters
4.0	2.7	3.9	6.95
3.8	3.9	3.75	5.5	9.4
3.6	4.8	4.8	7.3	12.3
3.4	5.6	5.75	9.1	15.2
3.2	6.4	6.75	11.0	18.0
3.0	7.2	7.5	13.15	20.7
2.8	7.9	8.25	15.3	23.6
2.6	8.35	8.8	17.1	26.2
2.4	8.5	9.3	18.0	

These experiments corroborate our conclusion that acids combine stoichiometrically with proteins if the hydrogen ion concentration is properly taken into consideration, that is, equal quantities of hydrogen ions combine at the same pH of the protein solution with a given mass of originally isoelectric protein no matter whether the acid added is a strong or a weak acid.

Figure 8 shows that when 100 c.c. of aqueous solution of 1 gm. of originally isoelectric gelatin contain different amounts of HCl the amount of gelatin chloride formed increases at first rapidly with increasing amounts of HCl. At pH 2.6 the curve tends to

become asymptotic to the axis of abscissæ, which means that practically all the gelatin has combined with HCl, so that any further addition of HCl will not increase the amount of gelatin in combination with acid. It was of theoretical importance to find whether, if the amount of HCl is increased, the combination curve continues to be parallel to the axis of abscissæ. This gap in our knowledge was filled by Dr. Hitchcock who used the writer's method. Hitchcock's experiments were made with 1, 2.5, and 5 per cent solutions of originally isoelectric gelatin to ascertain the combination curve for HCl and gelatin, and the

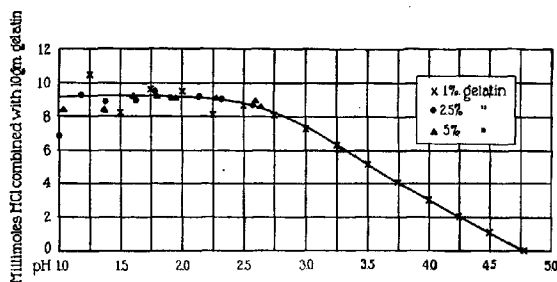


FIG. 9.—Combination curve of isoelectric gelatin with HCl, after Hitchcock. The curve shows that at about pH 2.5 the addition of more acid no longer increases the amount of gelatin chloride formed.

result which is given in Fig. 9 was that the curve is horizontal between pH 1.0 and 2.0, indicating that here the gelatin is all combined with acid. Lloyd and Mayes¹ had reported that the curve was irregular, but this was due to an error.

The maximum height of the curve in Fig. 9, 9.2 millimoles of HCl for 10 gm. of gelatin, indicates that a 1 per cent gelatin solution has a normality of 0.0092 with respect to its combination with HCl, or that the combining weight of gelatin is $\frac{10}{0.0092}$, or about 1,090. While the exact height of the maximum is still more or less uncertain, it is probable that this value of the combining weight is more nearly correct than the smaller values given by Procter,² Wilson,³ Wintgen and Krüger,⁴ and

¹ LLOYD, D. J. and MAYES, C., *Proc. Roy. Soc.*, vol. 93, p. 69, 1922.

² PROCTER, H. R., *J. Chem. Soc.*, vol. 105, p. 313, 1914.

³ WILSON, J. A., *J. Am. Leather Chem. Assoc.*, vol. 12, p. 108, 1917.

⁴ WINTGEN, R. and KRÜGER, K., *Kolloid-Z.*, vol. 28, p. 81, 1921.

Wintgen and Vogel,¹ because the calculation involves simpler and more probable assumptions. Moreover, the earlier workers did not have ash-free or isoelectric gelatin at their disposal.²

Similar experiments were made by the writer with casein prepared after the method of L. L. Van Slyke and J. C. Baker,³ who described in 1918 a method for preparing "pure casein" from skimmed milk, which consisted

in the gradual addition of acid and its immediate distribution through the mass of milk without causing coagulation of casein at the point where the acid first comes into contact with a portion of the milk. This result can be accomplished by introducing the acid below the surface of the milk with high-speed mechanical stirring. After standing under gentle stirring for 3 hours with acidity just below the point of casein coagulation, addition of acid is continued slowly, accompanied as before by rapid stirring in order to obtain the particles of casein coagulum in the finest possible state of division.

The coagulated casein is then centrifuged and after repeated washings is found free from Ca and inorganic P. As Van Slyke and Baker point out, the pH of this casein coagulum is about 4.5 to 4.6, *i.e.*, it is slightly below the isoelectric point. The essential feature of Van Slyke and Baker's method therefore consists in slowly bringing the milk or casein solution approximately to the pH of the isoelectric point of casein. The writer has shown that gelatin gives off all ionogenic impurities at the isoelectric point and Van Slyke and Baker's experiments show that the same method works also with casein. The casein prepared after Van Slyke and Baker's method is also free from albumin, since this latter protein is soluble at pH 4.5 or 4.7, and is, hence, removed from the insoluble isoelectric casein by washing.

In our experiments⁴ we used casein prepared after Van Slyke and Baker's method from skimmed milk and in addition from a "pure casein" of the market. Both preparations gave practi-

¹ WINTGEN, R. and VOGEL, H., *Kolloid-Z.*, vol. 30, p. 45, 1922.

² HITCHCOCK, D. I., *J. Gen. Physiol.*, vol. 4, p. 738, 1921-22.

³ VAN SLYKE, L. L. and BAKER, J. C., *J. Biol. Chem.*, vol. 35, p. 127, 1918.

⁴ LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 547, 1920-21.

cally the same result. In order to remove traces of fat from the casein, the latter was washed in acetone.

It is not possible to prepare 1 per cent casein solutions, except with a few acids, on account of the low solubility of the casein

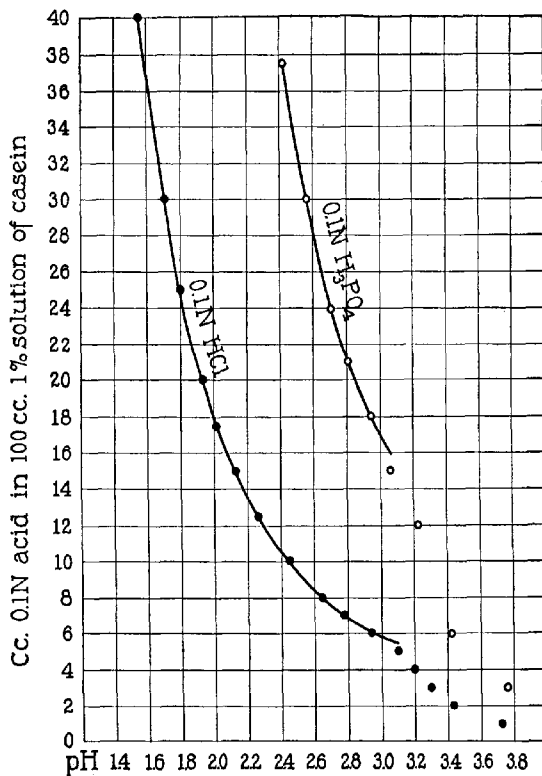


FIG. 10.—Ordinates represent the cubic centimeters of 0.1 N HCl or H_3PO_4 in 100 c.c. of 1 per cent casein solution. The abscissæ are the pH of the solution. Approximately three times as many cubic centimeters of 0.1 N H_3PO_4 as of 0.1 N HCl are required to bring 1 gm. of casein to the same pH.

salts with acids. It is, however, possible to compare casein chloride and casein phosphate in 1 per cent solutions. One gram

of isoelectric casein, prepared after Van Slyke and Baker, was put into 100 c.c. of aqueous solution containing 1, 2, 3, etc., cubic centimeters of 0.1 N HCl or 0.1 N H_3PO_4 . The pH of the casein solution was ascertained potentiometrically and the numbers of cubic centimeters of 0.1 N acid required to bring the 1 per cent casein solution to the same pH were plotted over the final pH of the casein solution as abscissæ. The casein chloride or casein phosphate is not completely soluble in a 1 per cent solution until the pH is about 3.0 or a trifle below. When too much acid is added, *i.e.*, when the pH is 1.6 or possibly a little above, casein precipitates out again from a 1 per cent solution.

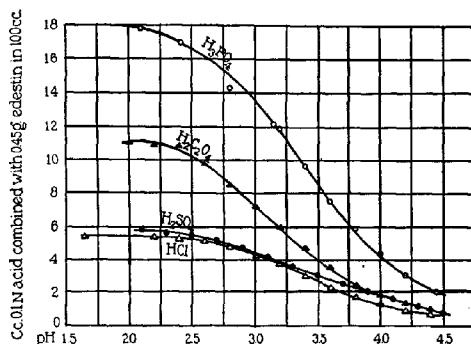


FIG. 11.—Amounts of 0.1 N acid combined with 0.45 gm. edestin in 100 c.c. Values for HCl, H_2SO_4 , and $\text{H}_2\text{C}_2\text{O}_4$ obtained by difference between titration curves with and without protein. Values for H_3PO_4 obtained by calculation. After Hitchcock.

Figure 10 gives the titration curves for HCl and H_3PO_4 , drawn out within those limits of pH within which the casein salts are soluble in a 1 per cent solution. The curves show that about three times as many cubic centimeters of 0.1 N H_3PO_4 as of 0.1 N HCl are required to bring 1 gm. of originally isoelectric casein in a 1 per cent solution to the same pH; or, in other words, H_3PO_4 combines with casein in molecular proportions, as should be expected if casein phosphate is a true chemical compound.

It was not possible to plot the corresponding curves for casein sulphate and casein oxalate, since these salts are too sparingly

soluble. This is true also for casein salts with other acids, *e.g.*, trichloroacetic acid.

Hitchcock has extended these investigations to two more proteins, namely, edestin and serum globulin.¹ Figure 11 gives the combination curves of HCl, H₂SO₄, H₂C₂O₄, and H₃PO₄² with 0.45 gm. of edestin in 100 c.c. His results confirm those of the writer obtained in the case of gelatin, albumin, and casein. There is little doubt left that such titration and combination curves will be found in the case of all proteins, and that therefore we may state that all proteins combine stoichiometrically with acids.

3. FURTHER TITRATION CURVES OF GELATIN WITH WEAK AND STRONG ACIDS

The amount of acid required to bring a 1 per cent solution of originally isoelectric protein, *e.g.*, gelatin, to the same pH varies with the nature of the acid. In Fig. 12 are given the titration curves of 1 gm. dry weight of originally isoelectric gelatin in a 100-c.c. aqueous solution for 0.1 *N* HCl, HBr, HI, H₂SO₄, sulphosalicylic acid; and also for the three weak monobasic acids, lactic, propionic, and acetic.

As was to be expected, the titration curves for HCl, HBr, and HI are practically identical with those for the two strong dibasic acids, H₂SO₄ and sulphosalicylic; or, in other words, the two strong dibasic acids combine in equivalent proportions with the protein. In the case of weak monobasic acids, higher concentrations of acid have to be added to bring the protein solution to the same pH. While 5 c.c. 0.1 *N* of all the strong acids must be contained in a 100 c.c. solution of originally 1 gm. dry weight isoelectric gelatin to bring the pH to 3.6, 10 c.c. of 0.1 *N* lactic acid are required for this purpose, and 65 c.c. of 0.1 *N* acetic or propionic acid. Yet the concentration of ionized gelatin is the

¹ HITCHCOCK, D. I., *J. Gen. Physiol.*, vol. 4, p. 597, 1921-22; vol. 5, p. 35, 1922-23.

² To get the true combination curve of a protein with a weak acid, such as H₃PO₄, it is necessary to consider the common ion effect of the anion of the protein-acid salt, which results in a repression of the ionization of the weak acid. The method of calculation is given by PAULI, W., "Kolloid-chemie der Eiweisskörper," p. 57, Dresden and Leipsic, 1920, and by HITCHCOCK, D. I., *J. Gen. Physiol.*, vol. 4, p. 597, 1921-22.

same in all acid solutions at the same pH, regardless of the nature of the acid.

On account of the enormous quantities required in the case of weak acids, it is not well possible to plot the quantity of acid in combination with a given mass of protein in the same way as

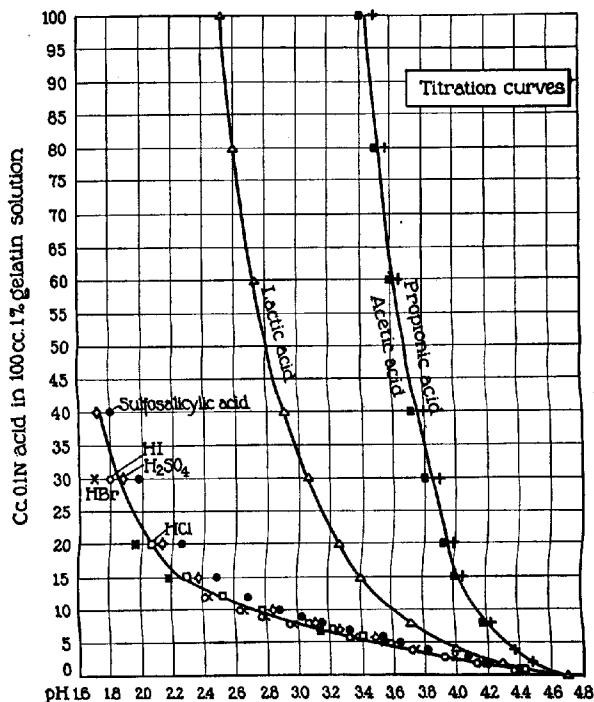


FIG. 12.—Titration curves of different acids with solutions containing 1 gm. dry weight of originally isoelectric gelatin in 100 c.c. The ordinates are the cubic centimeters of 0.1 N acid in 100 c.c. of gelatin solution; the abscissæ are the pH of gelatin solutions.

for HCl; but it will be shown in Chap. VII by an indirect method that the amount of anion combined with a given mass of protein in the same volume of solution is the same for a given pH no matter whether the acid is strong or weak.

4. TITRATION CURVES OF GENUINE PROTEIN WITH ALKALI

If the numbers of cubic centimeters of 0.1 N KOH, NaOH, Ca(OH)_2 , and Ba(OH)_2 are measured which must be contained

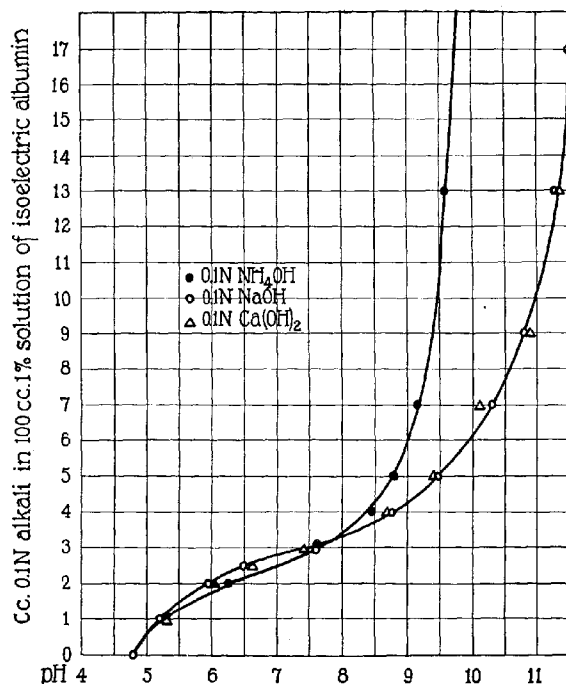


FIG. 13.—Curves representing the number of cubic centimeters of 0.1 N NH_4OH , NaOH, and Ca(OH)_2 required to bring 1 gm. of isoelectric, crystalline egg albumin in 100 c.c. solution to different pH. The curves for NaOH and Ca(OH)_2 are identical.

in 100 c.c. of a 1 per cent solution of originally isoelectric crystalline egg albumin to bring the solution to the same pH, it is found that these numbers are identical and that the values for the four bases lie in one curve. This means that Ca(OH)_2 and Ba(OH)_2

combine in equivalent proportions with crystalline egg albumin; *i.e.*, they combine with crystalline egg albumin in the same stoichiometrical way in which they combine with crystalloidal

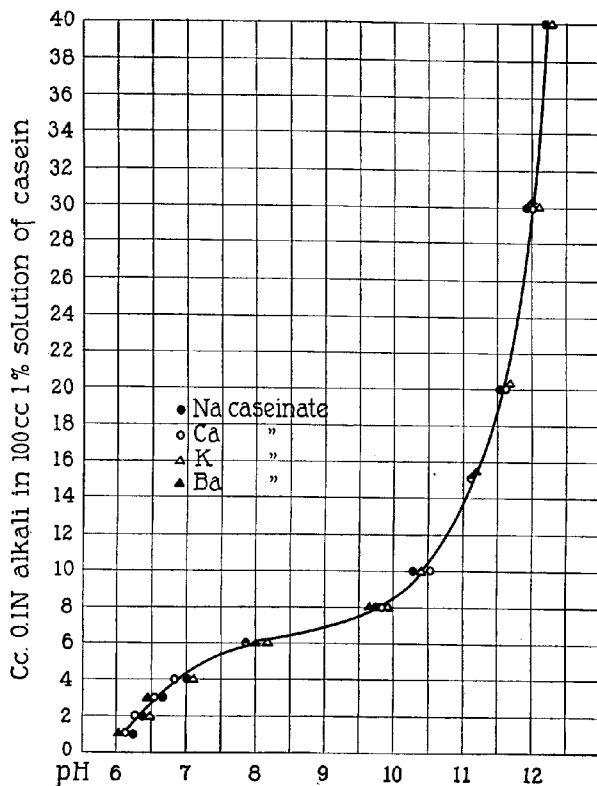


FIG. 14.—Ordinates are the cubic centimeters of 0.1 *N* NaOH, KOH, Ca(OH)₂, and Ba(OH)₂ in 100 c.c. of 1 per cent solution of casein. Abscissae are the pH of the solution. The curves for the four alkalis are identical, proving that Ba and Ca combine with casein in equivalent proportion.

acids. This is equally true for the combination of these bases with isoelectric albumin (Fig. 13), with casein (Fig. 14), and with

gelatin (Fig. 15). In this latter case the solution contained only about 0.8 gm. of originally isoelectric gelatin in a 100-c.c. solution.¹

These results were confirmed by Hitchcock for edestin and serum globulin.

The question may be finally raised: How many molecules of acid or alkali can combine with one molecule of protein? The

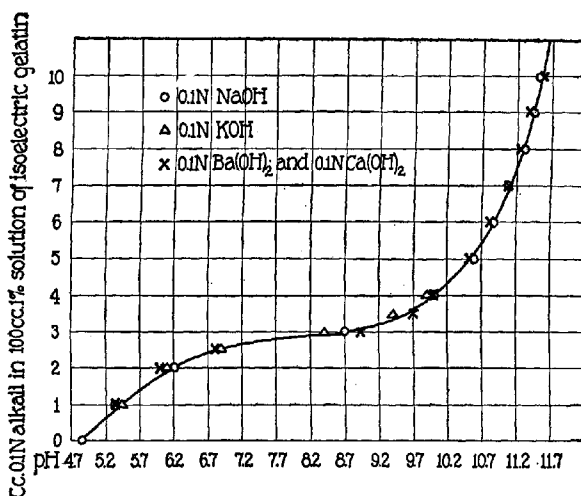


FIG. 15.—Curves for the number of cubic centimeters of 0.1 N NaOH, KOH, Ba(OH)₂, and Ca(OH)₂ required to bring the same mass of about 0.8 gm. of isoelectric gelatin in a 100-c.c. solution to different pH. All four curves are identical.

smoothness of the titration curves of isoelectric proteins with acids indicates that either only one or many molecules of a monobasic acid, *e.g.*, HCl, combine with one molecule of protein, since otherwise the curves could not be smooth. It is not probable that only one molecule of acid combines with one molecule of protein.

¹ LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 85, 1920-21.

Procter and Wilson,¹ have reached the conclusion that the equivalent weight of gelatin is 768, and Wintgen and Krüger² give the value as 839. Hitchcock found a higher number, namely, 1,120, as was stated above.³

According to recent analyses by Dakin,⁴ gelatin contains 1.4 per cent phenylalanine, which would give as the minimal molecular weight of gelatin 11,800. Hitchcock's value leads to the result that about ten (or a multiple of ten) molecules of a mono-basic acid combine with one molecule of gelatin.

Cohn and Hendry⁵ calculated from the titration curve of casein with sodium hydroxide a combining weight of about 2,100 for casein. Dakin found 1.7 per cent of tryptophane in casein and according to this the molecular weight of casein must be 12,000 (or a multiple thereof). This would indicate that six (or a multiple of six) hydroxyl ions combine with one molecule of casein. Cohn and Hendry point out that the calculation of the molecular weight of casein from sulphur and phosphorus contents agrees also with the molecular weight estimated from the titration curves.

Minimal molecular weight of casein calculated from tryptophane (average).....	12,800
Minimal molecular weight of casein calculated from phosphorus $\times 3$	13,116
Minimal molecular weight of casein calculated from sulphur $\times 3$	12,654
Equivalent combining weight of casein for sodium hydroxide $\times 6$...	12,600

All these data are difficult to understand on any other assumption than that proteins combine stoichiometrically with acid and alkali and that we are dealing with true chemical combination.

It can be stated, as the result of all these titration and combination experiments, that the ratios in which acids and bases combine with proteins are identical with the ratios in which acids and bases combine with crystalloids. Or, in other words, the forces by which gelatin, egg albumin, and casein (and probably proteins in general) combine with acids or alkalies are the purely

¹ WILSON, J. A., *J. Am. Leather Chem. Assoc.*, vol. 12, p. 108, 1917.

² WINTGEN, R. and KRÜGER, K., *Kolloid-Z.*, vol. 28, p. 81, 1921.

³ HITCHCOCK, D. I., *J. Gen. Physiol.*, vol. 4, p. 733, 1921-22; vol. 6, p. 95, 1923-24.

⁴ DAKIN, H. D., *J. Biol. Chem.*, vol. 44, p. 499, 1920.

⁵ COHN, E. J. and HENDRY, J. L., *J. Gen. Physiol.*, vol. 5, p. 548, 1922-23.

chemical forces of primary valency. There is nothing mysterious about this fact, since proteins are built up from amino-acids which are true crystalloids, and which combine stoichiometrically with acids and alkalies, as shown by Tague¹ and by Eckweiler, Noyes, and Falk.² These latter authors showed that this is true also for dipeptides. It would be surprising if the proteins which are polypeptides should not also combine stoichiometrically with acids or alkalies.

The question may be raised: How can the fact that proteins combine stoichiometrically be reconciled with the statement that their solutions frequently contain aggregates of molecules? This latter fact led to the assumption of absorption at the surface of each micella, but without cogent reason. The protein micellæ which may exist in a solution of gelatin in water are not comparable with metallic spheres or oil globules in water, where the two phases are separated by a continuous boundary impermeable to the electrolyte in solution. When a 1 per cent gelatin solution sets to a gel, the equal distribution of the molecules of the gel in the water remains the same. The random orientation of the gelatin molecules in the solution may change to a more definite orientation in the gel, but the average distance between the protein molecules will probably not change. The interstices between the molecules remain the same, and since water, acid, or alkali diffuse freely through a gel, the protein molecules and protein ions remain as accessible to alkali or acid in the gel as are molecules or ions in true solution. The micellæ of gelatin in solution are submicroscopic particles of jelly and there is no reason why the reactions between gelatin and electrolytes should cease to be stoichiometrical even if the protein were entirely in the gel state.

The titration and combination curves given in this chapter show also why it is necessary to compare the relative efficiency of two kinds of ions of the same sign not only for the same concentration of the originally isoelectric protein but also for the same pH. The combination curves (Figs. 6, 8, 9, 11, 13, 14, and 15) show that, as long as only little acid or alkali is added to isoelec-

¹ TAGUE, E. L., *J. Am. Chem. Soc.*, vol. 42, p. 173, 1920.

² ECKWEILER, H., NOYES, H. M. and FALK, K. G., *J. Gen. Physiol.*, vol. 3, p. 291, 1920-21.

tric protein, at each pH only part of the mass of the protein present exists in the form of a salt, the rest exists as non-ionogenic protein. Only if enough acid (or alkali) is added is all the protein transformed into a salt. The combination curves show that at the same pH the same fraction of the protein present exists in the form of a protein salt. If it is desired to compare the relative efficiency of different ions in combination with a protein one must be sure that the concentration of originally isoelectric protein is the same in both solutions and that the fraction of protein which has combined with the two ions is the same. This is only true when the solutions of the protein salts to be compared have not only the same concentration of originally isoelectric protein but also the same pH.

There existed no reason for comparing the effects of different ions at the same pH as long as the titration and combination curves given in this chapter were not known. But the knowledge of these curves forces the experimenter to change his methods and to base all the comparisons of the influence of ions on the physical properties of proteins on protein solutions of equal hydrogen ion concentrations. It is also necessary to point out that these hydrogen ion concentrations must be calculated from measurements with the hydrogen electrode, and not from conductivity measurements. The activity coefficient, and not the conductivity ratio, is the quantity which determines chemical reactions.

It has been argued that certain proteins, *e.g.*, gelatin, are a mixture of two or more different proteins. This may or may not be true, though it is probably not true for crystalline egg albumin. It is, however, immaterial for the proof of stoichiometrical behavior of protein solutions whether we are dealing with one or a mixture of two proteins as it is for the proof of the stoichiometrical behavior of acid whether HCl or a mixture of HCl and HNO_3 is titrated with alkali. Moreover, the results are not confined to gelatin but can with equal accuracy be obtained with other proteins, such as crystalline egg albumin, casein, edestin, and serum globulin.

If the methods of measuring the hydrogen ion concentration of protein solutions had been introduced before the colloidal speculations had been developed, nobody would have thought of sug-

gesting that proteins do not combine stoichiometrically with acids and alkalies. Scientists would have realized from the first that since proteins are built from amino-acids, they should logically be expected to react with acids and alkalies in the same stoichiometrical way as do amino-acids or dipeptides. It is always a misfortune for the development of science if problems are taken up before the proper methods for their solutions exist, because it is part of human nature that the authors of premature speculations based upon faulty or inadequate methods try to defend their erroneous views, in preference to learning and applying the new methods.

A new proof that the combination of proteins with acids is true chemical combination, following the ordinary laws of classical chemistry, has recently been added by Hitchcock.¹ Deaminized gelatin was prepared by treating gelatin with nitrous acid, following the procedure of Skraup. Determinations were made of the total nitrogen in gelatin and in deaminized gelatin, by the Kjeldahl method, and of the amino-nitrogen in gelatin, by the Van Slyke method. It was found that the loss of total nitrogen in gelatin which had been deaminized by Skraup's method was greater than the amino-nitrogen originally present in the gelatin. Accordingly, the procedure of Skraup was modified by avoiding the application of heat in preparing the deaminized gelatin. The resulting product was found to have undergone a loss in total nitrogen exactly equal to the amino-nitrogen originally present, indicating that under these conditions the deaminizing reaction really consisted simply in the replacement of amino groups by hydroxyl groups.

In order to determine the combining capacity of deaminized gelatin for hydrochloric acid it was necessary to ascertain its isoelectric point. This was done by measurements of the osmotic pressure developed at different pH values, following the procedure used by Loeb with other proteins. The minimum of osmotic pressure, and hence the isoelectric point of the protein, was found to be at pH 4.0. Finally, the combining capacity of the protein for hydrochloric acid was determined by electrometric titration with the hydrogen electrode, following a procedure similar to that previously used with gelatin and other proteins.

¹ Hitchcock, D. I., *J. Gen. Physiol.*, vol. 5, p. 95, 1923-24.

It was found that the difference between the maximum combining capacities of gelatin and of deaminized gelatin for hydrochloric acid was approximately equivalent to the free amino-nitrogen originally present in the gelatin and removed in the deaminizing reaction. Thus, the work constitutes a new type of evidence that the reactions of proteins with acid are truly chemical and stoichiometric.

CHAPTER V

ELECTRICAL CHARGES AND THE STABILITY OF SUSPENSIONS AND EMULSIONS

1. THE ORIGIN OF THE CHARGES OF COLLOIDAL PARTICLES

In the colloidal literature it is frequently held that aqueous solutions of genuine proteins are diphasic systems like suspensions of clay or emulsions of oil in water, where the particles are prevented from coalescing or agglutinating by mutual electrostatic repulsion due to electrical charges on the surface of each particle. The idea that solutions of genuine proteins are diphasic systems goes back to the classical experiments of Hardy on suspensions of solid particles of boiled white of egg. He observed that these suspensions were least stable at the isoelectric point where the particles no longer migrated in an electric field, showing that they were no longer charged.¹ It was observed later when the isoelectric point of genuine proteins was determined by Michaelis and his collaborators that many genuine proteins, such as gelatin, casein, cdestin, etc., were also least soluble at the isoelectric point; and the inference was drawn that solutions of genuine proteins in water were also suspensions or emulsions. This inference went too far, since we shall see in the next chapter that the forces by which certain proteins are kept in solution are the same forces by which crystalloids, *e.g.*, amino-acids, are kept in solution. The solubility of genuine proteins in water is a minimum at the isoelectric point, because at the isoelectric point the proteins are non-ionized, the non-ionized molecules being less soluble than the ionized. Experiments made by Michaelis and Davidsohn on the solubility of amino-acids, which are true crystalloids, have shown that their solubility is a minimum at the isoelectric point.

It has been the opinion among many colloidal workers that the electrical charges by which solid particles are kept in suspension are due to the preferential adsorption of certain ions, by

¹ HARDY, W. B., *Proc. Roy. Soc.*, vol. 66, p. 110, 1900.

which the charges of the adsorbed ions are transferred to the suspended particle.

The electronic theory of matter, however, has led to the conception of the existence of intrinsic potentials at the surface of solids and liquids. These intrinsic potentials, which have been discussed for metals by Frenkel¹ and (though not under that name) for water by Lenard,² are due to the fact that there exists generally a stratification of oppositely charged elements at the surface. In other words, an electrical double layer exists at the surface of each solid and liquid due to forces inherent in the substance itself. In the case of solid metals this electrical double layer may be due to the fact that there is an excess of electrons at the free surface. The existence of this double electrical layer does not betray itself until the two strata are separated, which can be done by bringing two different metals into contact. Electrons will then move from that metal which in the Volta series is more positive (*i.e.*, where the electrostatic attraction of the nucleus of the atom for the electrons in the outermost shell is smaller) into that metal which in the series is less positive. When the two metals are separated each is left with a charge, the one negative (because it now carries an excess of electrons), the other positive (because it has lost electrons).

At the surface of water there exists also a double electrical layer, the outermost stratum of which is negatively charged, while the deeper layer is positively charged. As long as the two strata are not separated, the existence of the double electrical layer is not perceptible, but when particles are torn mechanically from the surface of the water, it is found that they are negatively charged when they are small, while they are not charged when they are larger. This was proved by Lenard in his experiments on waterfall electricity. Inasmuch as these effects occur even in a vacuum, Lenard concludes that the formation of the electrical double layer at the surface of water is due to forces inherent in the water itself. Applying Frenkel's term to water, we may say that water also has an intrinsic potential due to the formation of an electrical double layer at the surface in which the outermost stratum is negatively charged.

¹ FRENKEL, J., *Phil. Mag.*, vol. 33, p. 297, 1917.

² LENARD, P., *Ann. Physik*, vol. 47, p. 463, 1915.

When gas bubbles are suspended in water an electrical double layer, due to the forces inherent in the water, must of course exist around each bubble. When a separation of the two strata of the double layer occurs the bubble is left with an electrical charge; and the charge should be negative on the basis of Lenard's experiments. This was confirmed by McTaggart¹ in his experiments on the migration of the air bubbles in an electrical field, where it was found that such bubbles moved towards the anode. McTaggart concluded from this that the outermost stratum of the water surface contains an excess of hydroxyl ions and that the layer of water underneath contains an excess of hydrogen ions. Since electrolytes raise the surface tension of water and are for this reason, according to Gibbs, pushed below the surface, one might ask whether hydrogen ions do not raise the surface tension of water more than do hydroxyl ions; in that case it would follow that hydrogen ions are pushed deeper into the water than hydroxyl ions. There are no data available at present to decide this question. McTaggart found that the nature of the gas in the bubble in no way influences the result, as was to be expected if the formation of the double layer is due to forces inherent exclusively in the water itself.

When the gas bubble is replaced by particles of an indifferent substance, that is, a substance the molecules of which have little or no affinity for water, *e.g.*, droplets of pure oil or of collodion, the forces inherent in the water should continue to give rise to an electrical double layer. If the electrical double layer is again exclusively or mainly due to forces inherent in the water, these particles should be negatively charged. It has been known ever since the first experiments on cataphoresis were made that particles are generally negatively charged in water. McTaggart pointed out that this was to be expected if the superficial stratum of the electrical double layer which adheres to the gas bubble or particle has an excess of OH ions.

When the material of the solid particle has a considerable affinity for water, when, *e.g.*, the material of the particles is ionized, complications may arise, which will be discussed in the

¹ McTAGGART, H. A., *Phil. Mag.*, vol. 27, p. 297, 1914; vol. 28, p. 367, 1914.

second part of this volume and may for the time being be omitted from consideration.

What interests us is the influence of electrolytes dissolved in the water on the intrinsic potential of the latter. It is natural to expect that when electrolytes are added to water their ions will be distributed unequally between the two strata of the double layer at the surface. McTaggart found that salts with trivalent and tetravalent cations in sufficient concentration had a tendency to reverse the sign of charge of the gas bubble. Since the forces which regulate the distribution of the oppositely charged ions of the salt are in this case inherent in the water, it means that trivalent cations were forced in larger quantity into the surface layer. Though it is customary to speak of the adsorption of the trivalent ion by the gas bubble, this can only have a metaphorical meaning, since the trivalent cations went into the surface layer of the water not on account of forces of adsorption due to the gas molecules but on account of forces inherent in the water itself. Moreover, Lenard's waterfall electricity exists also when there is a vacuum above the water and it does not seem appropriate to suggest that hydroxyl ions are in this case adsorbed by the vacuum. It would also be purely metaphorical to suggest that the "adsorbed" ions transfer their charges to the vacuum or to the gas bubble.

It would be of fundamental importance to continue McTaggart's experiments on the cataphoresis of air bubbles in order to find out how the oppositely charged ions of electrolytes distribute themselves between the two strata of the double layer of water, when every possibility of adsorption is excluded. But the experiments on cataphoresis of air bubbles are difficult and the writer substituted experiments with collodion particles. Collodion particles have only a small influence on the ionic stratification of the surface of water with which they are in contact. Like the air bubbles, they are negatively charged. The P.D. between air bubbles and water was found by McTaggart to be 55 millivolts, while the writer found as maximal P.D. between collodion particles and water 70 millivolts.¹ Experiments on the influence of electrolytes on the P.D. between collodion particles and water might, therefore, possibly give an idea as to how the ions of electrolytes would distribute themselves at the surface of water under

¹ LOEB, J., *J. Gen. Physiol.*, vol. 5, p. 109, 1922-23.

the influence of the forces inherent in the water itself, though it is not certain that the ionic stratification is actually similar in both cases.

The P.D. between collodion particles and water can be calculated on the basis of observations on the mobility of single particles in an electrical field. For these measurements the microscopic method of Ellis¹ and Powis² was used with the improved apparatus of Northrop³ with non-polarizable electrodes. From the mobility in centimeters per second, per volt per centimeter $\times 10^{-4}$, it is possible to calculate the P.D. between collodion particle and water by multiplying by 14 (at a temperature of about 24°C.). This calculation is based on the Helmholtz-Lamb equation

$$\text{P.D.} = \frac{4 \eta v \pi}{K X},$$

where η is the viscosity of the solution, v the velocity of the particle in centimeters per second, K the dielectric constant of the solution, and X the potential gradient, *i.e.*, the drop in potential in E. S. U. per centimeter. The details for this calculation can be gathered from Burton,⁴ Ellis, Powis, or Northrop. The special details concerning the preparation of the suspension of collodion particles can be found in the writer's original papers.

It was found that the P.D. between the particles and water was a minimum when the water approached the point of neutrality. The particles remained negatively charged in the presence of either alkali or acid. When the concentration of acids or alkalies was about $M/512$, the maximal P.D. was reached, and upon a further increase of acid or alkali the P.D. dropped again. This is illustrated in Fig. 16, where the abscissæ are the concentrations of acid or alkali and the ordinates the P.D. in millivolts. The sign of charge of the particles being negative, the P.D. values

¹ ELLIS, R., *Z. physik. Chem.*, vol. 78, p. 321, 1911; vol. 80, p. 597, 1912.

² POWIS, F., *Z. physik. Chem.*, vol. 89, p. 91, 1914-15.

³ NORTHROP, J. H., *J. Gen. Physiol.*, vol. 4, p. 629, 1921-22.

⁴ BURTON, E. F., "The Physical Properties of Colloidal Solutions," 2d ed., London, New York, Bombay, Calcutta, and Madras, pp. 136-37, 1921.

above the zero line are negative. The line "Critical P.D." is that P.D. below which the suspensions are no longer stable.¹

It follows from these observations that both acids and alkalis make the particles more negative, and we must conclude that

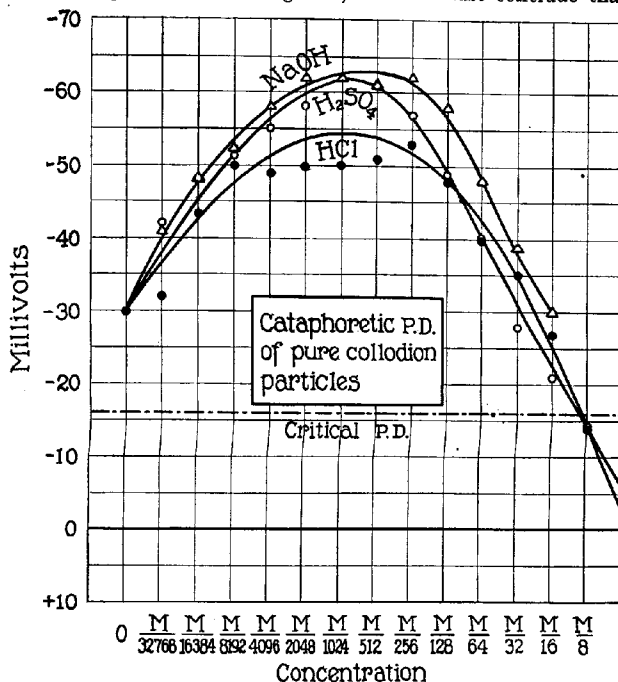


FIG. 16.—Influence of acid and alkali on the cataphoretic P.D. between collodion particles and water. The original pH of water was about 5.0. The abscissae are the concentrations of acids or alkali, the ordinates are the P.D. in millivolts. The collodion particles were negatively charged. The line marked "Critical P.D." (at 16 millivolts) is in this and the following figures the P.D. below which the collodion suspension is no longer stable.

the negative ions of either acid or alkali are driven into the outermost layer of water which moves with the particles, while the cations are driven deeper into the water.

¹ These and the following experiments were taken from LOEB, J., *J. Gen. Physiol.*, vol. 5, p. 109, 1922-23.

If we substitute salts for acids or alkalis we notice the same effect. The anions move into the most superficial layer of water, rendering the stratum which adheres to the colloidion particle more negative, provided the initial P.D. was low. The higher the valency of the anion of the salt the lower the concentration of the salt at which the maximal P.D. of about 70 millivolts is

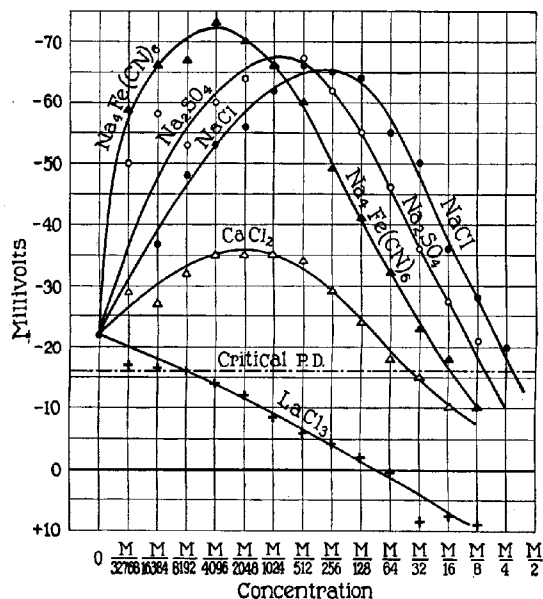


FIG. 17.—Influence of $\text{Na}_2\text{Fe}(\text{CN})_6$, Na_2SO_4 , NaCl , CaCl_2 , and LaCl_3 on the P.D. at pH 5.8. Addition of little salt with monovalent cation raises the P.D. to about 70 millivolts and the more rapidly the higher the valency of the anion. With CaCl_2 only a slight rise and with LaCl_3 no rise occurs in the concentrations used. In concentrations above $\frac{1}{64}$ LaCl_3 causes a reversal of the sign of charge of the particles.

reached. When more salt is added the P.D. drops again, owing probably to the fact that now more cations can get into the most superficial layer of the water. There is always a tendency for the cation to be pushed back deeper into the solution, but this tendency is perhaps the smaller the higher the valency of the

cation. Trivalent and tetravalent cations seem to be able to get into the most superficial stratum of the water readily, with the result that when a salt with trivalent cation like LaCl_3 is added the sign of the P.D. is reversed, the collodion particles assuming a positive charge and the water a negative charge.

Figure 17 gives the results of measurements of the P.D. between the collodion particles and solutions of five different salts, $\text{Na}_4\text{Fe}(\text{CN})_6$, Na_2SO_4 , NaCl , CaCl_2 , and LaCl_3 , all solutions having a pH of 5.8. This experiment was intended to illustrate the relative influence of the valency of the anions and cations on the cataphoretic P.D. The P.D. rose upon the addition of salts with univalent cation (Na) to a maximal value of about 70 millivolts, but the concentration required to reach this maximum was least for $\text{Na}_4\text{Fe}(\text{CN})_6$, a little higher for Na_2SO_4 , and still a little higher for NaCl . A further increase of the concentration of the salts depressed the P.D., the curves dropping rapidly (Fig. 17). The maximum was but slightly higher in the case of $\text{Na}_4\text{Fe}(\text{CN})_6$ than in the case of Na_2SO_4 , and but slightly higher in the case of Na_2SO_4 than in the case of NaCl .

Figure 17 shows also that the initial rise in the P.D. did not occur at all, or occurred at a very low concentration when the salt added was LaCl_3 , and that the rise was small when the salt added was CaCl_2 ; the maximal P.D. in the latter case was 36 millivolts at a concentration of $m/2,048$ of the salt. After the maximum was reached the curves dropped rapidly, and this drop was apparently due to the cation only. A comparison of the descending branches of the curves shows that to bring the P.D. down from the maximum (about 70 millivolts) to, e.g., 27.5 millivolts, the following molecular concentrations of the salts were required:

NaCl	$m/8$
Na_2SO_4	$m/16$
$\text{Na}_4\text{Fe}(\text{CN})_6$	slightly less than $m/32$
CaCl_2	between $m/128$ and $m/256$

This means that the depressing action of the three Na salts is almost the same for the same concentration of cations, regardless of the anion, while the depressing effect of CaCl_2 is between 16 and 32 times as great as that of NaCl . This leaves no doubt that the depressing effect is due to that ion which has the oppo-

site sign of charge to that of the collodion particle (or rather to that of the film of water moving with the particle), since the particle is negatively charged. This corresponds to the Hardy rule.

LaCl_3 depresses the P.D. at pH 5.8, even at low concentrations, so that at a molecular concentration of $\text{m}/64$ the P.D. is already zero (Fig. 17). With a further rise of the concentration of LaCl_3

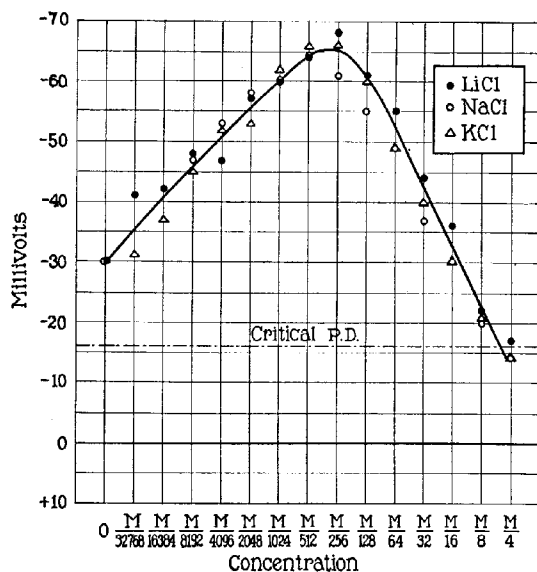


FIG. 18.—Influence of LiCl, NaCl, and KCl on the P.D. at pH 4.7.

the P.D. reverses its sign, the collodion particles assuming a positive and the water a negative charge. McTaggart observed the same reversal of the sign of charge of gas bubbles by trivalent cations. The cause of this reversal lies therefore primarily, if not exclusively, in forces inherent in the water itself.

The question arose whether other properties of the ion except its valency contribute to the depressing effect. The writer expected a difference in the depressing effects of LiCl, NaCl, and KCl. As a matter of fact, no difference in the influence of the

three salts on the cataphoretic P.D. was noticed, as Fig. 18 shows. If differences existed, they were within the limit of error in these experiments. These experiments were made at a pH of 4.7. Figure 19 shows that the influence of salts on the cataphoretic P.D. of collodion particles is about the same at a pH of 4.7 (Fig. 19) as at a pH of 5.8 (Fig. 17).

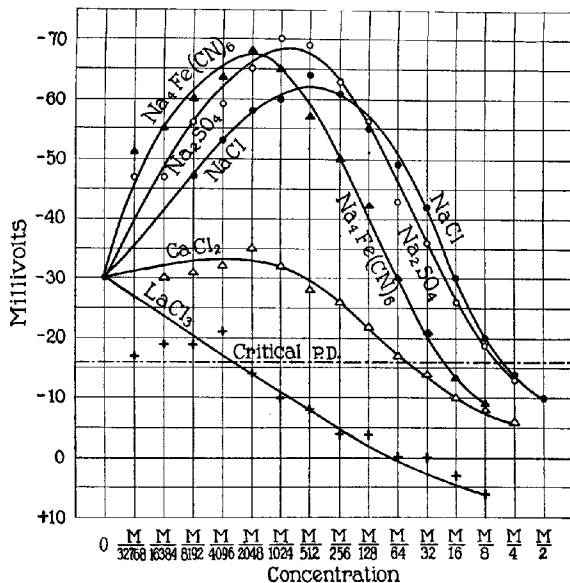


FIG. 19.—Similar to Fig. 17 except that the pH was 4.7.

When the experiments were made at a higher alkalinity, namely, in a $N/1,000$ KOH solution (Fig. 20), the P.D. was already about 60 millivolts when no salts were added, and the addition of salt could only raise the P.D. to its usual maximum of about 70 millivolts. This slight rise occurred in Na_2SO_4 and $\text{Na}_4\text{Fe(CN)}_6$, but to a less amount in NaCl . In concentrations of $M/256$ and higher, all the salts had only a depressing effect.

To bring the P.D. down to 35 millivolts in $N/1,000$ KOH, an approximate concentration of $M/16$ NaCl , of $M/32$ Na_2SO_4 , of

a little less than $M/64$ $\text{Na}_4\text{Fe}(\text{CN})_6$, and of approximately $M/1,500$ CaCl_2 was required. The depressing ion is therefore again the cation, as was to be expected, since the collodion particle, or rather the water film moving with it, is negatively charged.

When the experiments were made at higher hydrogen ion concentrations, *e.g.*, in $N/1,000$ HCl (Fig. 21), the particles had nearly

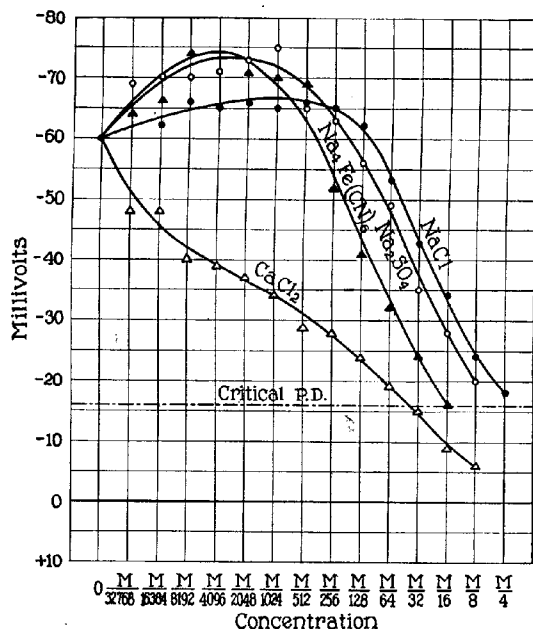


FIG. 20.—Influence of salts on the cataphoretic P.D. at pH 11.0. Without salt the P.D. was already near the maximum and hence the addition of salt had only a slight augmenting effect on the P.D.

their maximal charge without the addition of salt, since the P.D. was about 64 millivolts. Hence the addition of NaCl has no augmenting effect, while Na_2SO_4 has a slight augmenting effect to 72 millivolts. At concentrations above $M/256$ the salts depress the charge of the particles. Since the particles are always negatively charged, the depressing effect increases markedly

with the increasing valency of the cation, as was to be expected. To depress the charge to 27.5 millivolts, a concentration of $M/16$ NaCl , $M/256$ CaCl_2 , and $M/16,384$ LaCl_3 is required. In this acid solution LaCl_3 diminished the P.D. but did not bring about a reversal of the charge, perhaps for the reason that the original P. D. due to the acid was too high at the beginning.

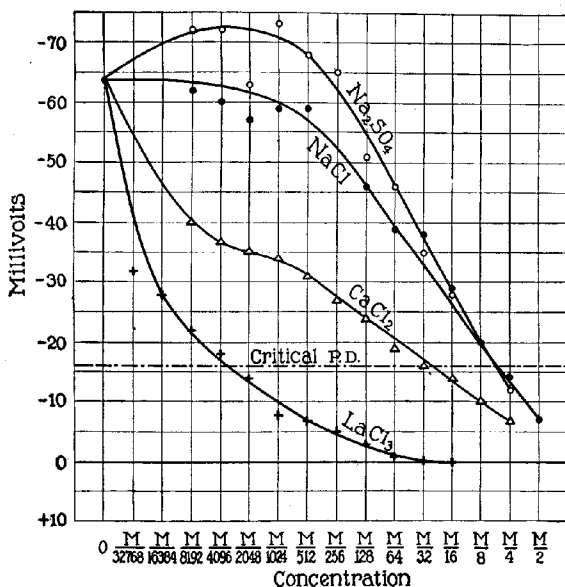


FIG. 21.—Influence of salts on the P.D. at pH 3.0. (See legend of Fig. 20.)

It is often stated that H and OH ions have a greater effect on the P.D. than other ions. This was not the case in our experiments. A comparison of Figs. 16 and 17 shows that HCl , H_2SO_4 , and NaOH act very much like NaCl or Na_2SO_4 on the P.D. The reason that in $N/1,000$ HCl or NaOH the addition of a salt no longer raises the P.D. is because the P.D. is already near the maximum before the salt is added, so that only the depressing effect of the salt becomes noticeable.

The idea that H and OH ions act more strongly than other monovalent ions is not true in the case of chemically inert substances like collodion. The statement that H ions act like Na ions on the cataphoretic P.D. of collodion particles is also borne out by the fact that acids do not depress the P.D. more than do Na ions, as a comparison of Figs. 16 and 17 shows. A reversal of the P.D. was observed when LaCl_3 was added, but not when HCl or NaCl was added to the solution.

All these experiments prove, however, that it is necessary to measure the hydrogen ion concentration of the solution, since the results otherwise are not strictly reproducible and comparable.

We therefore draw the conclusion that, as long as the concentration of electrolytes in water is low an excess of negative ions is always forced into the outermost layer of water which determines the sign of charge of the moving particle, while the cations are pushed deeper into the water; this is equally true for acids, alkalis, and salts. The forces which push the cations back into the water seem to diminish with increasing valency of the cation, so that if the cation is trivalent or tetravalent and the anion univalent it will happen that more cations will go into the outermost layer. In that case the sign of charge of the particle may be reversed. Because this reversal, however, occurs also when the collodion particle is replaced by an air bubble, it is quite possible that we are not dealing with an adsorption of ions by the collodion particle (or at least only to a minor extent), but that we are dealing with a modification of the intrinsic potential of the water due to an ionic stratification in the surface layer.

2. CRITICAL P.D. AND THE STABILITY OF SUSPENSIONS¹

It can be shown that the stability of a suspension of collodion particles free from protein depends on the cataphoretic P.D., since precipitation always occurs below the same critical cataphoretic P.D. of about 16 millivolts.

When the stock suspension of collodion particles was shaken up to produce an equal distribution of particles and 1 drop of this suspension was added to 10 c.c. of distilled water (of pH 5.8), the new suspension was milky when shaken up and remained so for several days. During this time the larger particles all settled

¹ LOEB, J., *J. Gen. Physiol.*, vol. 5, p. 109, 1922-23.

and only a cloudy gray suspension was left, which gradually, after the still further settling of larger particles, gave way to a bluish opalescent suspension which lasted for many weeks ("permanently"). In this case the settling was a slow process. When 1 drop of the stock suspension was put into 10 c.c. of an aqueous salt solution (also of pH 5.8), it was noticed that there existed a critical concentration of the salt, varying according to the nature of the salt, below which the suspension behaved as it did in distilled water, while in the next higher concentration a rapid, complete settling of the whole mass of collodion occurred in 12 hours or less, leaving not an opalescent but an entirely clear aqueous solution.

It was, therefore, comparatively easy to determine at which concentration the slow settling was replaced by a rapid settling caused by coalescence of the small particles into larger ones. It was found that at the concentrations of salts where the rapid settling occurred, the cataphoretic P.D. between particles and aqueous solution fell below the value of about 16 millivolts, regardless of the nature of the electrolyte used for precipitation. When the P.D. was above this value, the suspension was as stable as if no electrolyte had been added; and the stability was no greater at a P.D. of 60 or 70 millivolts, than at a P.D. of 50 or 25 millivolts.

Table IV gives the results of experiments on the precipitation of suspended particles of collodion by electrolytes. In one series of experiments the pH was 5.8, in a second 11.0, and in a third 3.0. In the second column of Table IV are given the minimal concentrations at which precipitation was observed, *i.e.*, in which the solution became completely clear in less than 18 hours, at about 20°C., while in the fourth column are found the maximal concentrations at which the suspensions remained "permanently" stable, *i.e.*, opaque for days and opalescent for weeks; in other words, where the salt caused no coalescence of particles. No attempt was made to locate the critical concentration more accurately than within the limits of concentrations given in the table, since it would probably not have been of any use in an attempt to define more sharply the real quantity of importance; namely, the critical P.D. between particles and solution. In column 3 are found the P.D. between particles and solution at

the minimal concentrations where precipitation occurred, and in the fifth column are found the P.D. of the maximal concentrations where the suspension remained stable.

TABLE IV.—CATAPHORETIC CHARGE AND STABILITY OF SUSPENSIONS OF PARTICLES OF COLLODION

1	2 Minimum concentration required for precipitation	3 P.D. in millivolts	4 Maximal concentration at which suspension remains stable	5 P.D. in millivolts
pH 5.8				
LiCl.....	m/2	(10)	m/4	17
NaCl.....	m/2	10	m/4	14
KCl.....	m/4	14	m/8	21
Na ₂ SO ₄	m/4	13	m/8	19
Na ₄ Fe(CN) ₆	m/16	13	m/32	21
MgCl ₂	m/16	11	m/32	15
MgSO ₄	m/16	15	m/32	19
CaCl ₂	m/32	14	m/64	17
LaCl ₃	m/2,048	14	m/4,096	21
pH 11.0				
NaCl.....	m/2	..	m/4	18
Na ₂ SO ₄	m/4	..	m/8	20
Na ₄ Fe(CN) ₆	m/16	16	m/32	24
CaCl ₂	m/32	15	m/64	19
pH 3.0				
NaCl.....	m/2	7	m/4	14
Na ₂ SO ₄	m/4	12	m/8	(lost)
CaCl ₂	m/32	16	m/64	19
LaCl ₃	m/2,048	14	m/4,096	18
H ₂ SO ₄	m/4	..	m/8	14

It may be pointed out that the precipitating action of acids like HCl or H_2SO_4 is of the same order of magnitude as that of Na salts but not of the order of magnitude of La salts. This agrees with the statement made in an earlier part of the paper that the acids act like salts with monovalent cation (*e.g.*, NaCl) on the P.D.

The average of all the P.D. values for the minimal concentrations at which precipitation occurred was 13 millivolts, while the average of all the P.D. at the concentrations at which the suspensions remained stable was 18.5 regardless of the pH. This suggests as the probable critical value for the P.D. where precipitation commences, about 16 millivolts. The actual P.D. evaluated from the mobility by cataphoresis is probably accurate only within ± 2 millivolts of this value, which explains some of the slight deviations from this value in Table IV.

These measurements confirm the conclusion reached by Powis¹ and by Burton,² as well as by Northrop and De Kruif,³ that there exists a critical P.D. for the stability of suspensions, this critical P.D. being about 16 millivolts for collodion particles in aqueous solutions. When the P.D. falls below this value the particles upon colliding are no longer repelled electrostatically but may adhere to each other and coalesce (*i.e.*, agglutinate or coagulate) into larger particles which rapidly sink to the bottom of the test tube. This coalescence of the colliding particles is due to forces of attraction between certain chemical groups of their molecules. If the P.D. is larger than 16 millivolts the particles will repel each other upon colliding with sufficient force to prevent coalescence. If this critical value is once exceeded the stability of the suspension is not increased when the charge is increased. The writer has noticed that there is no difference in the rate of settling of a suspension of the collodion particles when the charge varies between 20 and 70 millivolts.

Since the collodion particles are generally negatively charged it was to be expected that only the cation of the salt should be

¹ POWIS, F., *Z. physik. Chem.*, vol. 89, p. 186, 1914–15.

² BURTON, E. F., "The Physical Properties of Colloidal Solutions," 2d ed., London, New York, Bombay, Calcutta, and Madras, 1921.

³ NORTHROP, J. H. and DE KRUIF, P. H., *J. Gen. Physiol.*, vol. 4, pp. 639, 655, 1921–22.

responsible for the precipitation. This is corroborated by the fact that the precipitating efficiency of salts increases rapidly with the valency of the cation. Thus, for NaCl, CaCl₂, and LaCl₃ the precipitating efficiency measured by the reciprocal value of the minimal concentration required for precipitation (column 2, Table IV) is as 1:16:1,024. This valency effect is considerably greater than it would be if the P.D. responsible for the stability were due to the Donnan effect.

The question has often been raised whether that ion of a salt which has the same sign of charge as the colloidal particle will not counteract the precipitating action of the other ion. The molecular precipitating concentrations for NaCl, Na₂SO₄, and Na₄Fe(CN)₆ are $m/2$, $m/4$, and about $m/16$, respectively. In $m/2$ NaCl and $m/4$ Na₂SO₄ the concentration of cations is practically identical. If the anion had an inhibiting effect on precipitation, the concentration of Na₂SO₄ required for precipitation should be higher than $m/4$, which is not the case. The precipitating concentration of Na₄Fe(CN)₆ is even lower than that to be expected if only the Na ion acted. There exists no peptization effect of plurivalent anions in these experiments.

It is, however, possible to point out from our figures that such a peptization effect of a salt should be expected when the P.D. in pure water is below the critical value. If in that case the salt raises the P.D., the suspension will be stabilized.

3. CRITICAL P.D. AND THE STABILITY OF EMULSIONS

In the colloidal literature a distinction has been made between so-called emulsoids and suspensoids. This nomenclature was based on the assumption that the influence of electrolytes on the stability of emulsions was different from the influence of electrolytes on suspensions of solid particles, but Powis's experiments on oil drops show that this assumption is not correct.¹ Oil drops suspended in water are usually negatively charged and the maximal P.D. in Powis's experiments was 70 millivolts. They behaved in this respect like collodion particles, and we infer that the P.D. was due to forces inherent exclusively or largely in the water itself. It should be stated that Powis worked with pure oil, practically free from acid.

¹ Powis, F., *Z. physik. Chem.*, vol. 89, p. 91, 1914-15.

Figure 22 gives the influence of different salts on the P.D. of the droplets. The ordinates are the P.D. in volts, while the abscissæ are the cube root of the concentration in millimols per liter. The oil droplets were negatively charged like the collodion particles. The reader will notice that the addition of $K_4Fe(CN)_6$ increased the P.D. from about 46 to 70 millivolts, while KCl increased it from 46 to 61 millivolts. $BaCl_2$, $AlCl_3$, and $ThCl_4$ only depressed the charge and $AlCl_3$ and $ThCl_4$ reversed the sign

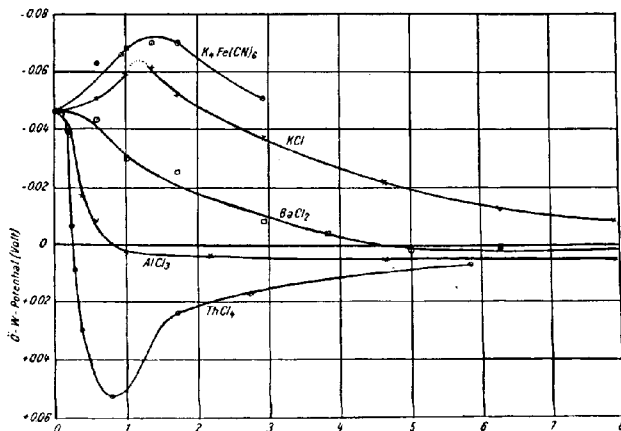


FIG. 22.—Influence of salts on the P.D. of droplets of oil in water, after Powis. Abscissæ are the cubic roots of the concentration of electrolytes in millimols per liter; ordinates the P.D. in volts.

of charge of the particles. Powis's Fig. 22 agrees essentially with Fig. 17, representing the observations on collodion particles. An investigation of the concentration of these salts required for flocculation of the oil emulsion showed that flocculation occurred when the P.D. fell below the critical value of 30 millivolts. Since the depressing action of salts rises with the valency of their cations, the precipitating action must also increase with the cation. This corresponds to Hardy's rule.

The conditions determining the stability of an oil emulsion are, therefore, the same as those determining the stability of suspended

particles of collodion or suspended typhoid bacilli or, perhaps, suspensions in general.

The reader will notice that in all these cases comparatively low concentrations of a salt suffice for flocculation and that the flocculating action of different salts increases rapidly with the valency of that ion of the salt which has the opposite sign of charge to that of the collodion particle or the oil droplet.

This will be of importance for the discussion of the question whether or not solutions of genuine proteins are emulsions.

The cataphoretic charges of particles with little or no affinity for water, such as oil drops or collodion particles, are not due to "adsorption" potentials, but are mainly the expression of the intrinsic potential of the water itself, *i.e.*, the ionic stratification of the surface of water, which is apparently determined by the forces inherent in the water itself.

The solid particle has also an intrinsic potential. If the surface layer of the suspended particles has a strong affinity for water, it is bound to modify or even to reverse the ionic stratification due to the forces inherent in the water alone.

CHAPTER VI

THE CRYSTALLOIDAL CHARACTER OF THE SOLUTIONS OF CERTAIN GENUINE PROTEINS IN WATER

The question at issue is: Are proteins kept in aqueous solution by the same forces which determine the solubility of crystalloids in water; or are they kept in solution by the electrical double layers which keep oil drops or collodion particles in suspension and which were discussed in the preceding chapter? We shall see that the answer differs for different proteins. The forces which keep crystalloids in solution are, according to Langmuir and Harkins, forces of secondary valency, *i.e.*, of attraction between the molecule of the solute (or rather certain groups of that molecule) and the molecules of water. This may be illustrated by the following quotation from Langmuir:

Acetic acid is readily soluble in water because the COOH group has a strong secondary valency by which it combines with water. Oleic acid is not soluble because the affinity of the hydrocarbon chains for water is less than their affinity for each other. When oleic acid is placed on water, the acid spreads upon the water, because by so doing *the COOH can dissolve in the water without separating the hydrocarbon chains from each other.*

When the surface on which the acid spreads is sufficiently large, the double bond in the hydrocarbon chain is also drawn onto the water surface, so that the area occupied is much greater than in the case of the saturated fatty acids.

Oils which do not contain active groups, as, for example, pure paraffin oil, do not spread upon the surface of water.¹

When particles are kept in suspension by double electrical layers, the fact betrays itself by two criteria, already emphasized in the preceding chapter, namely, first, that low concentrations of salts suffice to annihilate the P.D. and to bring about precipitation, and second, that the active ion of the salt has always

¹ LANGMUIR, I., *J. Am. Chem. Soc.*, vol. 39, p. 1850, 1917.

the opposite sign of charge to that of the particles. If these criteria are applied, it becomes obvious that aqueous solutions of certain genuine proteins, such as crystalline egg albumin or gelatin, are neither suspensions like those of collodion particles nor emulsions like those of pure oil in water.

It was noticed by all workers that enormous concentrations of salts are required to precipitate these proteins from their aqueous solutions, and it was found that the sign of charge of the active ion of the precipitating salt is not opposite to that of the protein ion to be precipitated. Eight-tenths per cent solutions of gelatin were prepared at three different pH, namely, 4.7 (isoelectric gelatin), 3.8 (gelatin chloride), and about 6.4 to 7.0 (Na gelatinate). The purpose was to find the molecular concentration of different salts, namely, $(\text{NH}_4)_2\text{SO}_4$, Na_2SO_4 , MgSO_4 , KCl , and MgCl_2 , required for precipitation. Table V shows that, regardless of the pH, the sulphates are better precipitants than the chlorides.

TABLE V.—MINIMAL MOLAR CONCENTRATIONS REQUIRED TO PRECIPITATE 0.8 PER CENT SOLUTIONS OF GELATIN

pH of gelatin solution	Approximate molecular concentration of salt required for precipitation				
	$(\text{NH}_4)_2\text{SO}_4$	Na_2SO_4	MgSO_4	KCl	MgCl_2
4.7.....	$1\frac{5}{16}$ M	$\frac{9}{8}$ M	$1\frac{9}{8}$ M	> 3 M	> 3 M
3.8 (gelatin chloride).....	$1\frac{3}{16}$ M	$\frac{9}{8}$ M	$\frac{3}{4}$ M	3 M	> 3 M
6.4 to 7.0 (Na gelatinate).....	$1\frac{5}{16}$ M	$\frac{9}{8}$ M	$\frac{9}{8}$ M	> 3 M	> 3 M

Table V shows that it was not possible to precipitate gelatin chloride or isoelectric gelatin or Na gelatinate by concentrations of MgCl_2 as high as 3 M, while sulphates were much better precipitants than the chlorides, regardless of the sign of charge of the protein ion, which was positive at pH 6.4, negative at pH 3.8, and zero at pH 4.7. If the protein particles were held in suspension by double electrical layers, MgCl_2 should have been a better precipitant for Na gelatinate than $(\text{NH}_4)_2\text{SO}_4$, while in reality the reverse was the case. We also have seen in the preceding chapter that when negatively charged collodion particles are

kept in suspension an $m/8$ $MgCl_2$ solution suffices already for precipitation.

The only logical conclusion is that the forces by which certain genuine proteins, such as gelatin, egg albumin, and others, are held in solution are not the forces due to electrical double layers surrounding each protein particle. Authors like Hardy ascribe the process of salting out of genuine proteins to some chemical alteration of the latter.¹ Abderhalden points out that this process seems to depend upon the presence of special amino-acid groups in the protein molecule, such as tyrosine or cystine.

Other authors who denied the crystalloidal nature of all protein solutions tried to escape from the difficulty by assuming that protein solutions were emulsions, so-called "emulsoids." Unfortunately for this assumption (which is not based on any fact and is merely an empty verbalism), emulsions of pure oils behave, according to Powis,² exactly like suspensions of collodion particles in regard to the influence of electrolytes on their agglutination, as was shown in the preceding chapter. To call solutions of genuine proteins emulsions or emulsoids does not remove the difficulties confronting those who assume that all genuine proteins are kept in solution by double electrical layers.

We are, therefore, compelled to consider the possibility that solutions of certain proteins in water do not differ from solutions of crystalloids. The only difficulty which confronts such an assumption are Hardy's observations on suspensions of denatured (boiled) white of egg, which showed a minimum of stability at the isoelectric point. Since the isoelectric particles of boiled white of egg do not migrate in the electrical field, Hardy correctly connected the lack of stability with the lack of charge. Now in this case there can be little doubt that the stability depends upon the double electrical layer surrounding each particle. When it was found that solutions of certain genuine proteins, like gelatin, also possess a minimal solubility at the isoelectric point, it was natural to see in this fact a confirmation of the idea that such proteins do not form crystalloidal solutions. The decision, therefore, depends on an investigation of the nature of the forces by which genuine proteins, such as gelatin, are kept in solution

¹ HARDY, W. B., *J. Physiol.*, vol. 33, p. 258, 1905-06.

² POWIS, F., *Z. physiol. Chem.*, vol. 89, p. 186, 1914-15.

at their isoelectric point. The writer has undertaken a series of experiments on isoelectric gelatin which seem to leave little doubt that these forces are the same which determine the solubility of crystalloids.¹

When solutions of 0.1, 0.2, etc. up to 1 per cent isoelectric gelatin were prepared in water of pH 4.7, put into test tubes, and left standing at sufficiently low temperature, it was found that only the tube with 0.1 gm. of gelatin in 100 c.c. remained perfectly clear, while the others became turbid, the turbidity increasing with the concentration of the gelatin. We can thus prepare a standard series of turbidities varying from 0.1 to 1.0 per cent gelatin solutions. Such solutions were kept for 24 hours in an ice chest (at about 2°C.) and when brought to room temperature could be used as a turbidity scale. The following table gives the scale of turbidity arbitrarily adopted:

Percentage of gelatin.....	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Degree of turbidity.....	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0

With this scale it became possible to determine the influence of salts on the solubility of 1 per cent solutions of isoelectric

TABLE VI.—INFLUENCE OF SALTS ON SOLUBILITY OF ISOELECTRIC PROTEIN
(The figures are the reciprocals of the solubility)

Concentration	1 M	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	M/8,192	M/16,384
NaCl.....	2.0	2.0	2.5	3.0	4.0	5.5	7.0	8.5	9.0	9.5	10.0	10.0			
LiCl.....	2.0	2.0	2.5	3.0	4.0	5.5	7.0	8.5	9.0	9.5	10.0	10.0	10.0		
KCl.....	2.0	2.0	2.5	3.0	3.5	4.5	6.5	8.5	9.0	10.0	10.0	10.0			
CaCl ₂	1.0	1.5	2.0	2.5	2.5	3.5	4.5	6.0	8.0	8.5	9.0				
MgCl ₂				2.0	2.5	3.0	3.5	5.0	6.0	7.0	8.5	9.0	9.0		
SrCl ₂				2.0	2.5	3.0	4.0	5.5	6.0	7.0	8.0	8.5	9.0	10.0	
BaCl ₂				3.5	3.5	4.5	6.0	7.0	8.5	9.0	9.5	10.0	10.0		
MgSO ₄			2.0	2.0	2.5	3.0	4.0	6.0	7.5	9.0	10.0	10.0	10.0	10.0	
Na ₂ SO ₄	5.5	2.5	2.0	2.0	2.5	3.0	5.0	7.0	8.0	9.0	10.0	10.0			
LaCl ₃				2.0	1.5	1.0	1.0	1.0	1.0	1.0	2.0	3.0	7.5	9.0	
CeCl ₃										Almost like water		1.0	1.0	2.0	5.0
Na ₂ Fe(CN) ₆										All clear like water			2.5		

¹ LOEB, J., *Arch. Néerland. physiol.*, vol. 7, p. 510, 1922.

gelatin. One gram of isoelectric gelatin was dissolved in 100 c.c. of different salt solutions, each having a pH of 4.7. Ten cubic centimeters of each of these 1 per cent solutions of isoelectric gelatin in different salts were put into a test tube, kept in the ice chest for 24 hours, and the turbidity was then determined with the turbidity scale. The salt solutions in which the 1 per cent isoelectric gelatin solutions were prepared all had a pH of 4.7. It was found that all the salts lowered the turbidity and the more so the higher the valency of either cation or anion of the salt. The results are given in Table VI. If we select for a comparison of the relative dissolving efficiency of different salts that concentration which is required to lower the turbidity from 10 (which is that of the 1 per cent solution of isoelectric gelatin without the presence of salts) to some lower value, *e.g.*, about 5, we find that the following concentrations of different salts are required:

LiCl, NaCl, KCl.....	m/32
MgCl ₂ , CaCl ₂ , SrCl ₂ , BaCl ₂ , Na ₂ SO ₄ , MgSO ₄ ...	between m/256 and m/128
LaCl ₃ , CeCl ₃ , Na ₄ Fe(CN) ₆	m/8,192 or less

In other words, uni-univalent salts double the solubility of isoelectric gelatin at a concentration of about m/32, while salts with one or both ions divalent have the same effect at a concentration of m/128 or m/256. Salts with one trivalent ion have that effect already at a concentration much lower than m/4,096. That we are dealing in this case with an increase of ordinary solubility can be proved by investigating the influence of these salts on the rate of solution of granules of solid isoelectric gelatin. It is found in this case that these salts lower the time required to dissolve a given mass of granules of isoelectric gelatin in agreement with the figures given in Table VII.

Doses of 0.8 gm. each of isoelectric gelatin of the same size of grain (going through mesh 30 but not through 50) were put into 50 c.c. of three different molar concentrations of certain salts, namely, m/1,024, m/512, and m/256, at a temperature of 35°C. and the time was ascertained which was required to dissolve completely this total mass of gelatin. The pH of all the solutions was 4.7. Table VII gives the results.

It is obvious that these salts increase the rate of solution of powdered isoelectric gelatin with increasing valency of both anion or cation.

TABLE VII.—TIME IN MINUTES REQUIRED TO DISSOLVE 0.8 GM. OF POWDERED ISOELECTRIC GELATIN AT 35°C.

	m/256	m/512	m/1,024
LiCl.....	57	70	76
NaCl.....	49	66	75
KCl.....	56	70	80
MgCl ₂	32	40	61
CaCl ₂	32	40	62
BaCl ₂	31	46	66
CeCl ₃	26	35	44
LaCl ₃	23		
Na ₂ SO ₄	34	46	60
Na ₄ Fe(CN) ₆	24	32	41

These experiments are open to the objection that perhaps these neutral salts confer an electric charge on isoelectric gelatin and that this increases the stability of the "emulsion." In order to test this possibility, cataphoresis experiments were made with suspended particles of isoelectric casein, gelatin, and denatured egg albumin, which permitted the evaluation of the charge of the protein particles. Since these experiments will be discussed in detail in the second part of this book, it may suffice to state that it was found that salts like NaCl, CaCl₂, or Na₂SO₄ do not confer any charge on the particles of isoelectric gelatin or any other isoelectric protein. This conclusion was confirmed by observations on the effects of these salts on anomalous osmosis¹ and on membrane potentials.²

All these facts prove, then, that the effects of NaCl, CaCl₂, and Na₂SO₄ on the clearing of isoelectric gelatin solutions are true solubility effects and not effects due to an increase of the charge of alleged droplets of so-called gelatin emulsion by the salt.

We must distinguish in the molecule of proteins between groups which have a greater affinity for water than for each other—which we will call "aqueous" groups (COOH or NH₂ groups)—and groups which have a greater affinity for each other than for water

¹ LOEB, J., *J. Gen. Physiol.*, vol. 4, p. 463, 1921-22.

² LOEB, J., *J. Gen. Physiol.*, vol. 4, p. 741, 1921-22.

—which we will call “oily” groups. If the forces of the aqueous groups prevail, the oily groups may be dragged into the water. With large molecules and when the oily groups are sparse, it may happen that when the oily groups of two adjoining molecules happen to come into contact the two molecules may adhere without noticeably diminishing the force with which the aqueous groups are held in the water. This seems to occur in solutions of gelatin. When the temperature is sufficiently high, the heat agitation will constantly tear the fusing gelatin molecules apart again; but if the heat agitation is slight, *i.e.*, the temperature sufficiently low, the two molecules may remain linked. In this way groups of adhering molecules will gradually form. This will result, first, in the formation of small pieces of jelly, and finally, in the whole mass solidifying to one coherent mass of jelly. The relative distribution of the molecules of gelatin in the jelly remains the same as it was in the solution, and the forces between the aqueous groups of the gelatin molecules and the molecules of water remain possibly the same; what changes is only the relative orientation and mobility of the individual gelatin molecules.

In the case of salting out or precipitation in all probability an entirely different change occurs, namely, a diminution of the force of attraction between the aqueous groups and the water. Such changes are brought about especially when sulphates are added to the solution of the protein.

That the relatively strong precipitating action of sulphates on solutions of genuine proteins is a phenomenon of ordinary solubility is indicated by the following experiments.¹

Powdered gelatin of not too small a size of grain (going through sieve 30 but not through sieve 60) was rendered isoelectric in the way described in Chap. II and about 0.8 gm. was put into 100 c.c. of each of a series of solutions of NaCl, CaCl₂, or Na₂SO₄, varying in concentration from M/4,096 to 2 M. The suspensions of the powdered gelatin were frequently stirred and the time required virtually to dissolve completely all the grains of powdered gelatin in suspension at 35°C. was measured. The ordinates in Fig. 23 are the solution times of isoelectric gelatin, and the abscissæ are the molecular concentrations of the salt used. It is obvious that NaCl and still more CaCl₂ increase the rate of solution of

¹ LOEB, J., and LOEB, R. F., *J. Gen. Physiol.*, vol. 4, p. 187, 1921–22.

isoelectric gelatin in water, and the more the higher the concentration of the salts added. There exists, however, a striking discontinuity in the Na_2SO_4 curve. As long as the concentration of Na_2SO_4 is below $M/32$, it increases the solubility of gelatin, and the more so the higher the concentration. When, however, the concentration of Na_2SO_4 is above $M/32$, a further increase in this concentration diminishes the solubility of gelatin

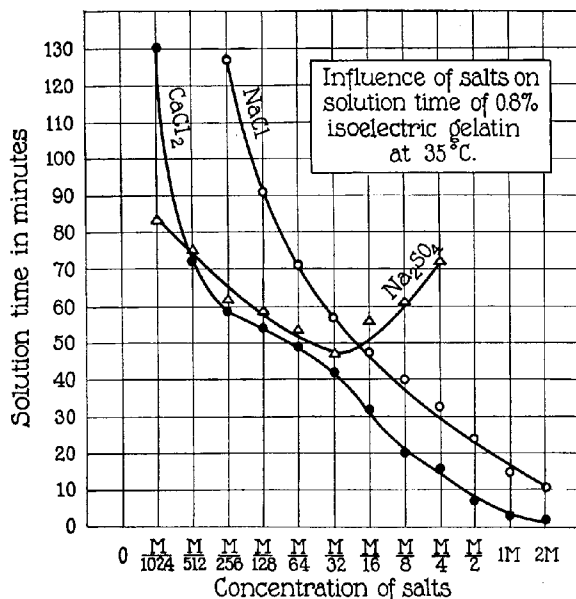


FIG. 23.—Influence of salts on the time required for the solution of 0.8 gm. of powdered isoelectric gelatin in a 100-c.c. salt solution at 35°C. and pH 4.7. Notice difference of curve for Na_2SO_4 and for CaCl_2 and NaCl .

and the more so the higher the concentration of Na_2SO_4 . $(\text{NH}_4)_2\text{SO}_4$ acts like Na_2SO_4 .

The curve for the solution time of powdered isoelectric gelatin in Na_2SO_4 or $(\text{NH}_4)_2\text{SO}_4$ suggests that we are dealing with two opposite effects, one of which leads to an increase in the rate of solution with increasing concentration of Na_2SO_4 . This effect

prevails at lower concentrations of the sulphates up to $M/32$. When the concentration rises above $M/32$, the second effect increases more rapidly than the dissolving action of the sulphates. This second effect, which diminishes the solubility, may or may not be a chemical effect resulting in a modification of the gelatin whereby its solubility is diminished. No such effect occurs in

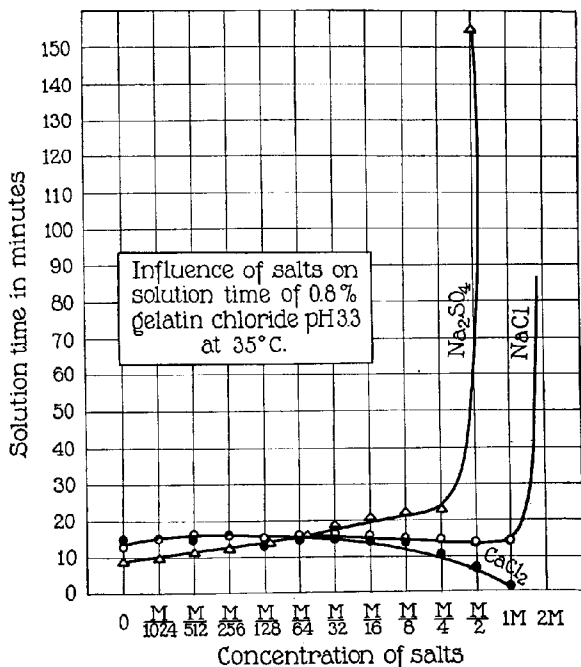


FIG. 24.—Influence of salts on solution time of 0.8 gm. of powdered gelatin chloride of pH 3.3 in a 100-c.c. salt solution at pH 3.3. The gelatin is no longer soluble beyond 1M NaCl.

the case of the chlorides. This suggests why sulphates are better precipitants than chlorides, although it remains for further investigation to show what makes sulphates act differently. But this seems to be a problem of the general theory of solution rather than of a theory of colloidal behavior.

While isoelectric gelatin is only sparingly soluble, gelatin salts are highly soluble. Doses of 0.8 gm. of powdered gelatin of pH of about 3.3 dissolve very rapidly in 100 c.c. of HCl of the same pH at 35°C. The addition of NaCl or CaCl_2 no longer increases the solubility, except for CaCl_2 in concentrations above $\text{m}/16$. Na_2SO_4 or $(\text{NH}_4)_2\text{SO}_4$ abruptly diminishes the solubility

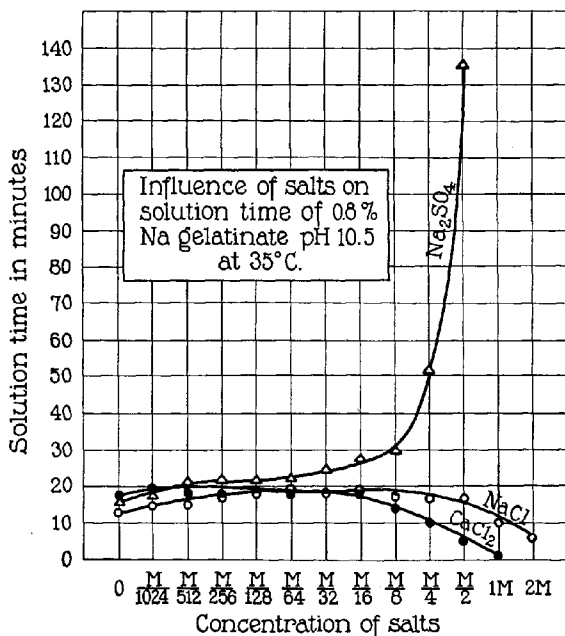


Fig. 25.—Influence of salts on solution time of 0.8 gm. of powdered Na gelatin in a 100-c.c. salt solution at pH 10.5.

at a concentration above $\text{m}/4$; and NaCl does so above a concentration of 1 M (Fig. 24).

Figure 25 shows the influence of the three salts on the solution time of Na gelatin of pH 10.5. Na_2SO_4 diminishes the solubility abruptly at a concentration above $\text{m}/8$, while both NaCl

and CaCl_2 increase the solubility of Na gelatin, NaCl in concentrations above $\text{M}/2$, and CaCl_2 in concentrations above $\text{M}/16$.

Wherever the solubility is diminished by a salt, we may possibly be dealing with a secondary effect of the salt on the constitution or configuration of the protein molecule. These facts explain the salting out which is no longer a valency effect but a specific effect of definite substances.

These experiments leave little doubt that the action of Na_2SO_4 is due to a diminution of ordinary solubility of gelatin.

If we put all these facts together, we must arrive at the conclusion that the action of salts on the stability of gelatin solutions supports the idea that gelatin forms crystalloidal solutions and the same is true for solutions of certain other genuine proteins, such as crystalline egg albumin. The reason that proteins with true crystalloidal solubility, such as gelatin or crystalline egg albumin, have a minimum of solubility at their isoelectric point is because the solubility of a protein increases with its degree of electrolytic dissociation and this is a minimum at the isoelectric point. This had already been discussed by Michaelis in the first edition of his book.¹ Michaelis and Davidsohn have shown that the solubility of a crystalloidal amphoteric electrolyte, namely, *p*-aminobenzoic acid, is a minimum at the isoelectric point.² The same could probably be shown in the case of all amino-acids. The fact that the solubility of proteins is a minimum at the isoelectric point can, therefore, not be used as an argument that they do not form true solutions.

This conclusion is further supported by the important experiments of Cohn.³ On the basis of the law of the constancy of the solubility product, Cohn and Hendry⁴ have given proof that casein in alkali forms a true or molecularly dispersed solution. These authors have shown that casein forms a well defined soluble disodium compound, and that solubility was completely determined by (a) the solubility of the casein molecule, and (b) the concentration

¹ MICHAELIS, L., "Die Wasserstoffionenkonzentration," pp. 41 ff., Berlin, 1914.

² MICHAELIS, L. and DAVIDSOHN, H., *Biochem. Z.*, vol. 30, p. 143, 1910. "Die Wasserstoffionenkonzentration," p. 44, Berlin, 1914.

³ COHN, E. J., *J. Gen. Physiol.*, vol. 4, p. 697, 1921-22.

⁴ COHN, E. J. and HENDRY, J. L., *J. Gen. Physiol.*, vol. 5, p. 521, 1922-23.

of the disodium casein compound. From the study of systems containing the protein and very small amounts of sodium hydroxide it was possible to determine the solubility of the casein molecule, and also the degree to which it dissociated as a divalent acid and combined with base. Solubility in such systems increased in direct proportion to the amount of sodium hydroxide they contained. The concentration of the soluble casein compound varied inversely as the square of the hydrogen ion concentration, directly as the solubility of the casein molecule, and as the constants K_{a_1} and K_{a_2} defining its acid dissociation.

CHAPTER VII

THE VALENCY RULE AND THE ALLEGED HOFMEISTER SERIES

(A) OSMOTIC PRESSURE

Crystalloidal properties of proteins, such as solubility (and possibly cohesion and others), are very probably affected not only by the valency but also by the chemical nature of the ion. Thus, isoelectric gels of gelatin are dissolved more rapidly in NaI than in NaCl. Casein is less soluble in trichloroacetic acid than in nitric acid, and less in nitric than in hydrochloric acid. When we are dealing, however, with the specifically colloidal behavior of proteins, as shown in the influence of electrolytes on swelling, osmotic pressure, a certain type of viscosity, and also membrane potentials, only the valency of the acid or alkali added to isoelectric protein affects these properties; while the chemical nature of the ion plays no rôle (except indirectly, through a possible influence on cohesion or solubility). This fact, which we will call the valency rule, is of paramount importance for the theory of colloidal behavior, as will become evident in the second part of this book.

The fact to be proved, namely, the valency rule, is contrary to the statements current in colloid chemistry, according to which the chemical nature of the ion is of as much importance as the valency. As already stated in the first chapter, the ions have been arranged in series, the so-called Hofmeister series, according to their relative influence on swelling, viscosity, and osmotic pressure of proteins, but it can be shown that these series are in this case largely the result of the same methodical error which had prevented the recognition of the fact that acids and alkalis combine with proteins stoichiometrically, namely, the failure to measure the hydrogen ion concentration of the protein solutions. If we wish to compare the relative efficiency of two ions, *e.g.*, Cl and CH_3COO , on the osmotic pressure or viscosity of

protein solutions, it is absolutely necessary to do so at the same pH and the same concentration of originally isoelectric protein. If this is done, it will be found that the Hofmeister series have practically no real significance in the case of swelling, osmotic pressure, and viscosity of proteins, and that essentially only the valency, not the specific nature of the ion in combination with the protein, influences the specifically colloidal properties, such as osmotic pressure, swelling, and a certain type of viscosity.

In Chap. IV it was seen that at the same pH three times as many cubic centimeters of 0.1 N H_3PO_4 as of HNO_3 are in combination with 1 gm. of originally isoelectric gelatin in 100 c.c. of solution. From this it follows that the anion of gelatin phosphate is the monovalent ion H_2PO_4 and not the trivalent anion PO_4 . It follows likewise from the combining ratios discussed in Chap. IV that the anion of oxalic acid in combination with protein below $\text{pH} = 3.0$ is the monovalent anion HC_2O_4 , while at pH above 3.0 the oxalic acid dissociates to an increasing degree as a dibasic acid, forming a divalent anion C_2O_4 with protein. The same must be true, *mutatis mutandis*, for all weak dibasic or tribasic acids, e.g., citric, tartaric, or succinic acids, namely, that at pH below 4.7 they form protein salts with chiefly monovalent anions. It follows also, from the combining ratios, that the salt of a protein with a strong dibasic acid, as H_2SO_4 , must have a divalent anion, e.g., SO_4 . On the basis of our valency rule, we should therefore expect that the osmotic pressure of 1 per cent solutions of originally isoelectric gelatin with different acids of the same pH should be identical for all gelatin salts with monovalent anion; in other words, 1 per cent solutions of gelatin chloride, bromide, nitrate, or phosphate should all have about the same osmotic pressure and the same viscosity at the same pH; and the same should be true for swelling; while gelatin sulphate, which has a bivalent anion, should have a much lower osmotic pressure, viscosity, or swelling. We will show first that this is true for the osmotic pressure of protein solutions.

The simple method of R. S. Lillie¹ was employed for the measurement of the osmotic pressure of gelatin solutions. Collodion bags of a volume of about 50 c.c. were cast in Erlenmeyer flasks, assuming the shape of the latter. These were prepared in

¹ LILLIE, R. S., *Am. J. Physiol.*, vol. 20, p. 127, 1907-08.

a uniform way, as follows: Collodion (Merck, 275 grains of ether per ounce; 27 per cent alcohol, U. S. P. IX) was used. Erlenmeyer flasks of a volume of about 50 c.c. were rinsed with 95 per cent alcohol and then filled to the neck with the collodion solution. After the flask was filled with collodion, the latter was allowed to pour out slowly from the flask, which was rotated slowly by hand during this process. The process of rotating the flask and pouring out the collodion was timed to occupy exactly 2 minutes. Then the Erlenmeyer flask, which was now empty except for a film of collodion adhering to the inside of the glass wall, was allowed to dry for exactly 2 minutes at room temperature. It was then put under the faucet and tap water was allowed to run in in a gentle stream for 5 minutes. The collodion film formed inside the flask could be pulled out, being an exact cast of the flask. These collodion bags were closed, with the aid of rubber bands, by a conical rubber stopper which was perforated to allow a glass tube to be pushed through. The collodion bag was filled with the solution of protein with the aid of a small funnel, all air bubbles were removed, and the glass tube was pushed into the bag to serve as a manometer. The bag was then put into a beaker usually containing 350 c.c. of water, having the same pH as the protein solution. The surface of the stopper was so adjusted that it lay in the surface of the water in the beaker and the glass tube (or manometer) was pushed a little deeper into the bag, so that at the beginning the level of the protein solution was about from 20 to 30 mm. above that of the water in the outside beaker.

The water diffused from the outside beaker into the protein solution and the column of liquid in the manometer rose to a maximum which was usually reached in about 6 hours or possibly less. It must be taken into consideration that two changes in pH will occur in these experiments which affect the osmotic equilibrium. The one change is due to the Donnan equilibrium, which was referred to in the first chapter. The other change is due to the influence of the CO_2 of the air in the outside solution, and this influence is especially disturbing when alkaline solutions are used. It must also be borne in mind that in these experiments the protein solution is also usually diluted through the entrance of water into the collodion bag. In later chapters

measures will be mentioned by which these sources of error can be avoided or diminished.

One per cent solutions of originally isoelectric protein were made, each solution containing a certain amount of an acid or of

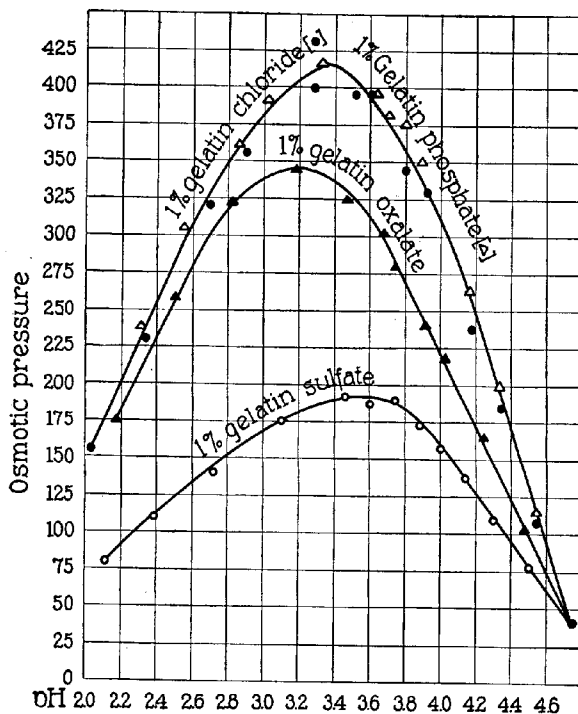


FIG. 26.—Influence of pH and valency of anion on osmotic pressure of solutions of different gelatin-acid salts. The osmotic pressure is a minimum at the isoelectric point, pH 4.7, rises with the addition of acid until pH is 3.4, and then drops upon the addition of more acid. The curves for gelatin chloride and gelatin phosphate are identical.

alkali to give it a definite pH. In each case the collodion bag containing the gelatin solution was dipped, as described, into a beaker containing 350 c.c. of water of originally the same pH as that of the protein solution used. On account of the Donnan

equilibrium this equality of pH in the inside and outside solutions was not retained, the pH rising higher inside than outside in the case of solutions of gelatin-acid salts. The observations lasted usually for 1 day but the level of liquid in each manometer was recorded at first every 20 or 30 minutes and the values recorded the next day were used to plot the curves in Fig. 26. The osmotic equilibrium was usually established in about 6 hours. The experiments were carried on in a thermostat at a temperature of 24°C.

Figure 26 gives the curves of the osmotic pressure for solutions of originally 1 per cent dry weight isoelectric gelatin with four different acids, HCl, H₂SO₄, oxalic, and phosphoric acids.¹ The abscissæ are the pH of the gelatin solutions after osmotic equilibrium was established, *i.e.*, at the end of the experiment. The pH was always determined potentiometrically. The reader will notice that the four curves have a number of characteristic features in common. The osmotic pressure is, in all cases, a minimum at the isoelectric point, namely, at pH = 4.7; it rises with increasing hydrogen ion concentration (or diminishing pH), and the curves all reach a maximum at about pH = 3.4. When the hydrogen ion concentration rises still further (or with a further drop in pH) the curves for the osmotic pressure of the solutions of the four gelatin salts diminish almost as steeply as they rose on the other side of the maximum. It may be noticed in passing, that Pauli² and Manabe and Matula³ speak of a maximum in the viscosity curves of albumin at a pH of about 2.1. It will be observed that the maximum for osmotic pressure lies at a much higher pH, namely, at about pH 3.4, and that at pH 2.1 the curves are at a low level again, not much above that of the isoelectric point. This form of the curves of osmotic pressure when plotted as a function of pH of the protein solutions is very characteristic and invariable.

The main point, however, which interests us in this connection is the proof of the valency rule. The titration curves show that in the case of gelatin phosphate as well as of gelatin chloride the

¹ LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 691, 1920-21.

² PAULI, W., "Kolloidchemie der Eiweisskörper," Dresden and Leipsic, 1920.

³ MANABE, K. and MATULA, J., *Biochem. Z.*, vol. 52, p. 369, 1913.

anion is monovalent, H_2PO_4 and Cl . The valency rule demands that the osmotic pressures of the two salts should be identical, and a glance at Fig. 26 shows that this is the case. The anion of gelatin oxalate should also be essentially monovalent for pH below 3.0 and we see that the descending branch of the oxalate curve, from pH 3.0 and below, practically coincides with the descending branch of the curve for gelatin chloride and phosphate. For pH above 3.0 the curve for the osmotic pressures of gelatin oxalate is slightly lower than the curve for gelatin phosphate and gelatin chloride, as the theory of electrolytic dissociation demands, since for pH above 3.0 oxalic acid dissociates electrolytically more and more like a dibasic acid the higher the pH. Hence, at about pH 3.4 the majority of the anions of gelatin oxalate are monovalent, but a certain small percentage is divalent. For this reason the curve for gelatin oxalate is at pH 3.4 or for higher pH not quite as high as that for gelatin chloride or phosphate. This is in strict agreement with the titration curves in Fig. 7.

The titration curves in Fig. 7 show also that H_2SO_4 forms a divalent anion in combining with gelatin and we notice that the maximum of the osmotic pressure curve at pH 3.4 is less than one-half that of the osmotic pressure curve for gelatin chloride or gelatin phosphate at the same pH.

These results are then in full agreement with the titration experiments if we assume that only the sign and the valency of the ion with which the protein is in combination determine the osmotic pressure of the protein salt formed, while the nature of the ion has either no effect or, if it has any effect, the latter must be so small that it escapes detection.

If the Hofmeister series were correct, we should have expected that the curve for the osmotic pressure of gelatin phosphate should have been of the order of that of gelatin sulphate or even lower, instead of being equal to that of gelatin chloride; and the same should have been true for the curve for gelatin oxalate.

The writer has repeated these experiments so often that there can be no doubt about the correctness of the result.

If the valency rule is correct, it must be shown that all acids with monovalent anions give curves identical with that for HCl , and all acids with divalent anion must give curves identical with

that for H_2SO_4 . Seven acids with monovalent anions were used for this test, namely, HCl , HBr , HI , HNO_3 , acetic, lactic, and propionic acids. The influence of these acids on the osmotic pressure of gelatin solutions containing 1 gm. dry weight of originally isoelectric gelatin in a 100-c.c. solution is plotted in Fig. 27. The abscissæ are the pH, the ordinates the osmotic pressure

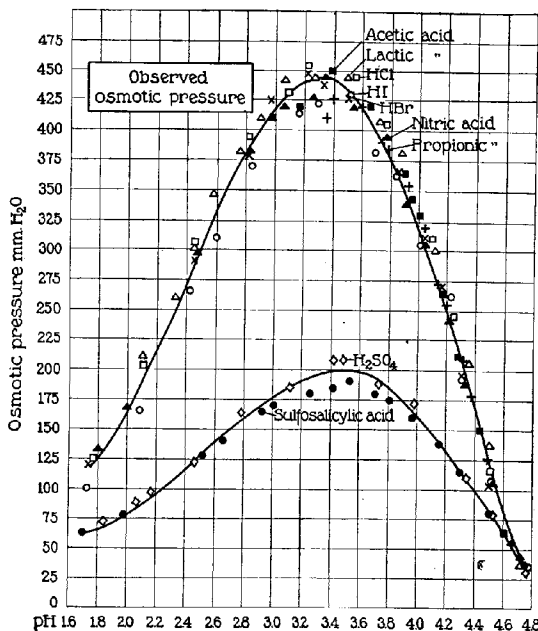


FIG. 27.—Proof of valency rule for the influence of acids on the osmotic pressure of gelatin solutions. The influence of seven monobasic acids on the osmotic pressure of gelatin solutions is the same and about twice as high as that of the two dibasic acids.

in terms of millimeters of a column of water. The values for all seven acids with monovalent anion lie on one curve (within the limits of experimental accuracy). Two strong dibasic acids were used, H_2SO_4 , and sulphosalicylic. The values of the effect of these two acids also lie on one curve, which is a little less than

half as high as that for the acids with monovalent anion. These experiments, therefore, prove that only the valency and not the chemical nature of the anion of the acid influences the osmotic pressure of gelatin solutions.¹

Figure 28 gives a comparison of the effect of three weak dibasic and tribasic acids, namely, succinic, tartaric, and citric, on the

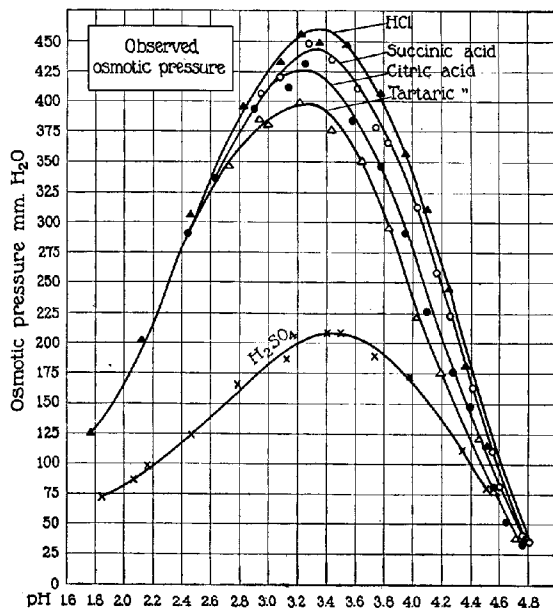


FIG. 28.—Influence of weak dibasic and tribasic acids on the osmotic pressure of gelatin solutions.

osmotic pressure of a gelatin solution containing 1 gm. dry weight originally isoelectric gelatin in a 100-c.c. solution. As was to be expected, the descending branches of the curves for the effect of these three acids on the osmotic pressure of the gelatin solution are identical with the descending branch of the HCl curve, since these weak dibasic and tribasic acids dissociate like monobasic

¹ LOEB, J. and KUNITZ, M., *J. Gen. Physiol.*, vol. 5, p. 665, 1922-23.

acids at a pH below 3.0; while above pH 3.0 the curves for the weak dibasic or tribasic acids are slightly lower than the curve for HCl in the order of their relative strength. This latter fact means that at a pH > 3.0 the second ion begins to be split off and the more the stronger the acid. Thus, in the case of the weak succinic acid only a very small percentage of molecules dissociate as a dibasic acid and the same may be said for citric acid, while a greater percentage of tartaric acid molecules dissociate as dibasic acid between pH 3.0 and 4.7. These experiments might almost

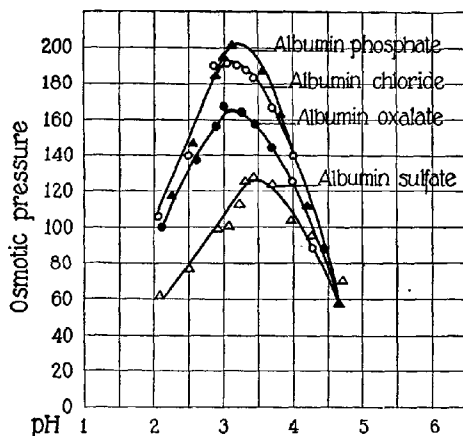


FIG. 29.—Osmotic pressure of different albumin-acid salts. The ordinates indicate the osmotic pressure (in millimeters of 1 per cent albumin solution); the abscissæ are the pH. All solutions are 1 per cent in regard to isoelectric albumin. The curves for albumin chloride and albumin phosphate are identical.

be used as a criterion for the order of dissociation of weak dibasic and tribasic acids.

These experiments leave no doubt that only the valency and not the chemical nature of the anion of the acid determines the influence of the acids on the osmotic pressure of the gelatin solution.

The valency rule holds true not only for the osmotic pressure of gelatin solutions, but of many, if not all, protein solutions. Experiments with 1 per cent solutions of originally isoelectric

crystalline egg albumin confirm the valency rule also for this salt.¹ The abscissæ in Fig. 29 are the pH of the albumin solutions determined at the end of the experiment, the ordinates the osmotic pressure after equilibrium was reached. The acids used were HCl, H₂SO₄, oxalic acid, and H₃PO₄. The reader notices again that the osmotic pressures are a minimum at the isoelectric point, that they reach a maximum at pH a little above pH 3.2, and that they then drop again.

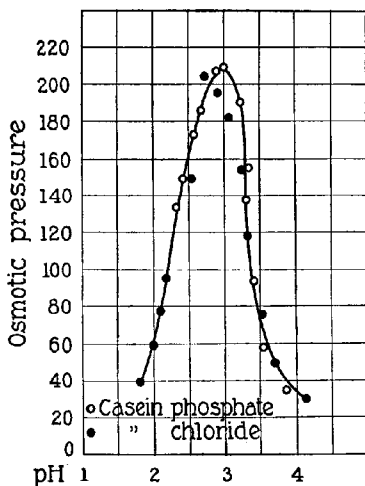


FIG. 30.—Osmotic pressure of 1 per cent solutions of casein chloride and casein phosphate as function of pH. The two curves are almost identical.

The four curves confirm the valency rule. The curves for albumin chloride and albumin phosphate are practically identical, that for albumin sulphate is almost but not quite half as high as that of phosphate, and the curve for oxalate is at the maximum a little lower than that for chloride.

The valency rule holds also for casein-acid salts.² Since casein oxalate and sulphate are too sparingly soluble, we can only compare the osmotic pressures of casein phosphate and casein

¹ LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 85, 1920-21.

² LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 547, 1920-21.

chloride. The curves for the osmotic pressures of these two salts are alike if plotted over the pH, as Fig. 30 shows. The maximal osmotic pressure lies at pH of about 3.0.

Hitchcock measured the effect of HCl, H_3PO_4 , $\text{H}_2\text{C}_2\text{O}_4$, and H_2SO_4 on the osmotic pressure of solutions of edestin and confirmed the writer's results.

There is, then, no doubt that the curves for the osmotic pressures of gelatin, crystalline egg albumin, casein and edestin obey the valency rule, and show no appreciable influence of the nature of the ion, except that of the sign of charge and valency.

In the older experiments in which the hydrogen ion concentrations were not measured, the action of weak acids led the investigators into error. In the Hofmeister series it is generally contended that acetic acid acts like sulphuric acid and not like hydrochloric or nitric acids. This is due to the fact that the investigators compared the effects of different acids at equal molecular concentrations instead of comparing the effects of different acids at the same pH. If the latter procedure is followed it is found that acetic acid acts like HCl and not like H_2SO_4 , as shown in Fig. 27.

The idea that the valency of the ion in combination with a protein is the chief if not the only factor which influences its osmotic pressure is corroborated by measurements of the osmotic pressure of metal gelatinates. We had shown in Chap. IV that $\text{Ca}(\text{OH})_2$ and $\text{Ba}(\text{OH})_2$ combine with gelatin in equivalent proportions and that, hence, the ion in combination with gelatin in these cases is the bivalent cation Ca or Ba. The experiments showed that Li, Na, K, and NH_4 gelatinates have about the same osmotic pressure at the same pH and the same concentration of originally isoelectric gelatin; while under the same conditions Ba and Ca gelatinates have an osmotic pressure less than one-half of that of the metal gelatinates with monovalent cation.¹ The same is true in the case of the metal salts of crystalline egg albumin. Figure 31 shows that the curves for the osmotic pressure of NH_4 and Na albuminate are about the same for the same pH, while that for Ca albuminate is about half as high.

¹ LOEB, J., *J. Gen. Physiol.*, vol. 3, pp. 85, 547, 1920-21.

Similar results were obtained in the case of the osmotic pressure of metal caseinates.

All experiments agree that only the valency of the ion with which a protein is in combination determines its osmotic pressure, while the chemical nature of the ion seems to have no influence.

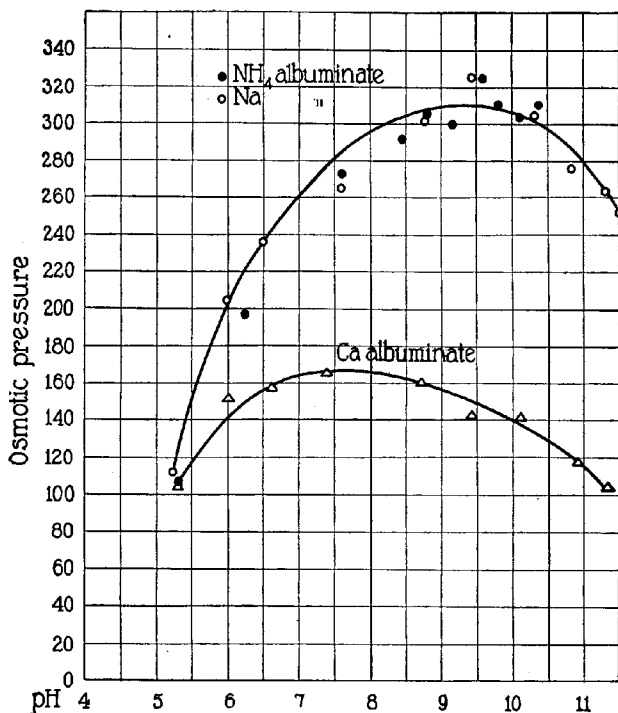


FIG. 31.—Curves of the osmotic pressure of NH_4 , Na, and Ca albuminate at different pH. The curves for NH_4 and Na albuminate are practically identical.

This fact is of the greatest significance, since it was to be expected if colloidal behavior is due to the Donnan equilibrium. The writer may state that this valency rule was found before he was aware of the fact that the influence of electrolytes on the osmotic

pressure of protein solutions could be derived from the Donnan equilibrium.

(B) SWELLING

It is generally stated in colloidal literature that solid blocks of gelatin swell more in chlorides, bromides, or nitrates than in water and that they swell less in citrates, acetates, tartrates, phosphates, and sulphates than in water. The author of this statement is Hofmeister,¹ who was a pioneer and who cannot be blamed for not considering the hydrogen ion concentration of his solutions, about which nothing was known at the time of his experiments. In Hofmeister's experiments gelatin blocks were put into salt solutions of a high concentration, and the differences in the effects observed in different solutions were slight.

He states that even sugar solutions have a dehydrating effect, like certain salts, and this fact alone should have warned chemists that his experiments could not be used for conclusions concerning the specific effects of ions on the physical properties of colloids. As far as the writer knows, the discrimination between "hydrating" and "dehydrating" ions originated from these experiments.

It is often asserted that Hofmeister's ion series for swelling have been confirmed by other authors. Thus on page 373 of Zsigmondy's book, "*Kolloidchemie*" (2d edition), the following statements are made in support of this impression:

Wo. Ostwald, who compared the efficiency of different acids, found that swelling diminishes in the acids in the following order, $\text{HCl} > \text{HNO}_3 > \text{acetic acid} > \text{sulphuric acid} > \text{boric acid}$. Fischer has shown that the acid and alkali swelling of gelatin as well as that of fibrin is diminished by the addition of salt, and that chlorides, bromides, and nitrates have a less dehydrating action than acetates, sulphates, or citrates. We have here a similar series as in the case of the precipitation of proteins by alkali salts, although the order does not agree entirely.

The writer is inclined to interpret Ostwald's and Fischer's experiments differently from Zsigmondy, since both authors ignored the hydrogen ion concentration of their gels. Our experiments have shown that it is necessary to base conclusions concerning the relative efficiency of ions on experiments with

¹ HOFMEISTER, F., *Arch. expil. Path. Pharmacol.*, vol. 28, p. 210, 1891.

equal hydrogen ion concentration of the protein solution or gel. By ignoring this postulate Ostwald succeeded only in proving that acetic and boric are weaker acids than nitric, but not that the acetate and borate anions have a greater depressing effect on the swelling of gelatin than NO_3 ; and Fischer succeeded only in proving that citrates and acetates are buffer salts which when added to a solution of a strong acid diminish its hydrogen ion concentration, but not that the acetate and citrate anions have a greater depressing effect on the swelling of gelatin than Cl or NO_3 . Both authors erroneously ascribed the effects of variation of pH to an effect of the nature of the anion. The Hofmeister series of ion effects on swelling have, in reality, never been confirmed.

If we wish to study the specific effects of ions on the swelling of gelatin we must proceed from isoelectric gelatin, bringing it to different pH by different acids or alkalis and then compare the swelling at the same pH of the gel. We shall see in the second part of this book that swelling depends on the concentration of protein ions in the gel and this depends on the pH inside the gel at equilibrium. For this reason, the effects of different acids must be compared at the same pH of the gel. If this is done, it is found that only the valency of the anion of an acid influences the swelling of gelatin; and the influence of the valency of the acid is similar to that on osmotic pressure.

The most accurate method for determining the degree of swelling of gelatin is by weighing the gelatin at the beginning and at the end of the experiment. The earlier experimenters used large blocks of gelatin for the weighing experiment, but in that case it requires days before the maximum of swelling is reached and in the meantime some of the gelatin has probably been dissolved, and the nature of the anion may have a considerable influence on solubility. It is, therefore, obvious that experiments on the swelling of blocks of gelatin cannot well be used for theoretical conclusions. We used finely powered particles of gelatin of a definite size of grain, namely, particles which went through a sieve with mesh of $\frac{1}{30}$ but not through mesh $\frac{1}{60}$ of an inch. It was found that such particles reach the maximal swelling in 2 hours. By making the experiment at 15°C ., the loss by solution of the gelatin was considerably less than 5 per cent. After the acid had acted for 2 hours at 15°C ., on the gelatin, the latter was put on a

filter, the solution allowed to drain off, and the weight of the gelatin was determined. In this way better results could be obtained than with the older method of using a solid block or by estimating the swelling from the volume of the powdered gelatin.

A large quantity of powdered gelatin of the size of grain as stated was brought to the isoelectric point in the way described in Chap. II. Eight grams of the wet isoelectric powdered material contained 1 gm. dry weight of isoelectric gelatin. This mass of powdered isoelectric gelatin in equilibrium with water served as stock material and was kept moist in an ice chest at about 3°C. Equal portions of 8 gm. each of the stock gelatin, each containing about 1 gm. dry weight of isoelectric gelatin, were put into beakers and allowed to stand for about 18 hours in a moist chamber at 15 to 16°C. The 8-gm. portions of gelatin were then added each to 150 c.c. of H₂O containing various amounts of 0.1 *N* acids of 15°C. and allowed to stand, with frequent stirring, for 2 hours at 15°C. The gelatin was then removed from the outside solution by filtration through cotton wool in weighed funnels. The liquid was allowed to drain off completely and the weight of gelatin was determined. The gelatin was afterwards melted at 50°C., cooled to 25°C., and its pH was measured electrometrically. All operations, except pH measurements, were carried out in a constant-temperature room at about 15°C. A control consisting of 9 c.c. of 0.1 *N* HCl per 150 c.c. of H₂O was used with each series. The pH of the gelatin gel of the control was 3.19 and the weight of the control varied mainly between 34 and 36 gm., the extreme variations being 32.3 and 37.8 gm.

Figure 32 shows the influence of the four acids, HCl, H₃PO₄, oxalic, and sulphuric, on the swelling of gelatin. The curves for HCl and H₃PO₄ are identical, the curve for oxalic acid is slightly lower—the difference being less below pH 3.0 than above—and the curve for H₂SO₄ is considerably lower than that for the others. The influence of these four acids on swelling is entirely similar to that on osmotic pressure, since only the valency but not the chemical nature of the anion determines this influence.

The correctness of the valency rule is confirmed by the following experiments. Figure 33 gives the results with different acids. The abscissæ are the pH of the gel at the end of the experiment, while the ordinates are the weight of the gelatin at the end

of the experiment. All the values for the influence of the six monobasic acids, HCl, HBr, HI, HNO_3 , propionic, and lactic acid, on swelling lie on the same curve within the limits of the

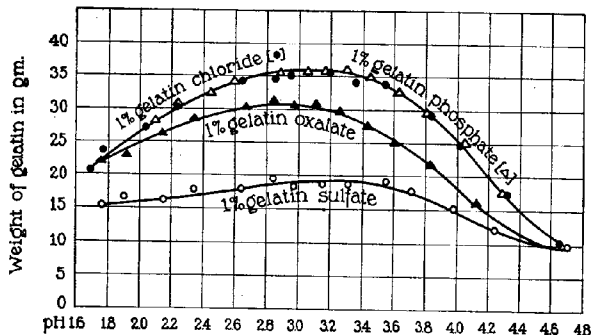


Fig. 32.—Influence of pH and valency of anion on swelling of gelatin.

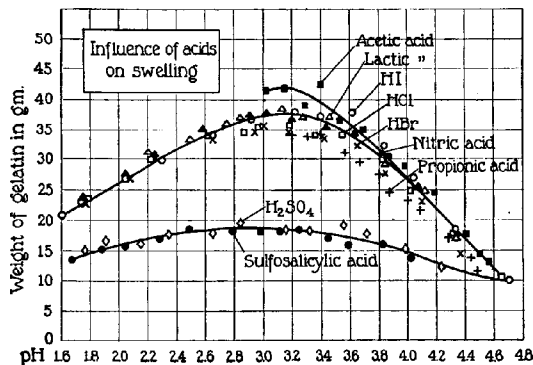


Fig. 33.—Proof of valency rule for the influence of acids on the swelling of gels of gelatin. The influence of the seven monobasic acids is (aside from slight secondary effects of acids, presumably on the cohesion of the gel) the same and considerably higher than that of the two dibasic acids.

accuracy of the experiments with a maximal weight of about 36 gm., which is inside the variations for the controls with HCl referred to. Only acetic acid gives a slightly higher maximal

value of about 42 gm. at pH 3.2. The abnormal behavior of acetic acid does not occur in either membrane potentials or osmotic pressure, where the effects are due to isolated gelatin ions. The suspicion is, therefore, justified that the excessive effect of swelling is due to a diminution of the cohesion of the gel caused by the high concentration of acetic acid required to bring the pH to 3.2 or to 3.0.

On the other hand, the strong dibasic acids, H_2SO_4 and sulphosalicylic acid, also act alike, causing a maximal weight of only 18 gm., which is about one-half of the maximal weight of the

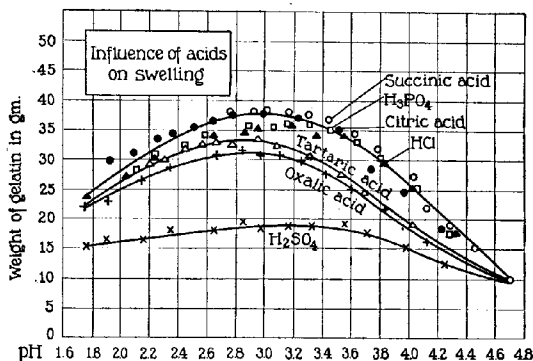


Fig. 34.—Influence of weak dibasic and tribasic acids on swelling.

gelatin under the influence of HCl. This ratio of 1:2 for dibasic and monobasic acids is about the same as that observed for the valency effect of anions in the case of osmotic pressure. The maximum lies at a pH of about 3.0 to 3.2 of the gel.

These experiments show that only the valency but not the chemical nature of the anion of the acid influences the swelling of gelatin.

Figure 34 shows the effect of weak dibasic and tribasic acids on swelling. From what has been said concerning the electrolytic dissociation of these acids, it is obvious that their effect on swelling is also as clearly a confirmation of the valency rule as is their action on membrane potentials and on osmotic pressure.

Figure 35 gives the curves for the action of alkalis on the swelling of gelatin. The curves for Li, Na, K, and NH_4 gelatinates of the same pH are practically the same, except that the values for NH_4OH are irregular for pH above 8.5, possibly on account of the fact that the concentration of NH_4OH required to bring gelatin to such pH is rather high. The main fact is that the ratio of the maximal swelling of gelatin salts with bivalent cation, like Ca or Ba, is considerably less than that of gelatin salts with monovalent cation, like Na, K, or NH_4 .¹ This agrees

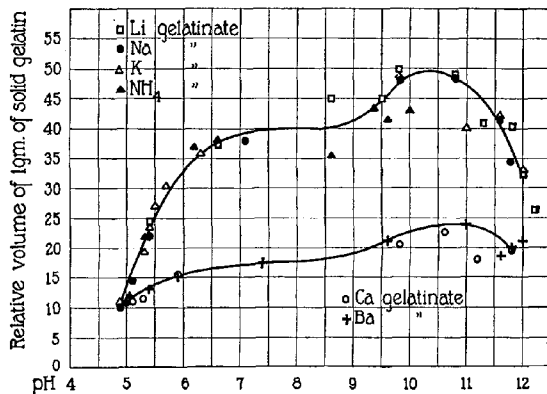


FIG. 35.—Curves for the effect of different bases on swelling. Those for LiOH , NaOH , KOH , and NH_4OH are practically identical and about twice as high as those for $\text{Ca}(\text{OH})_2$ and $\text{Ba}(\text{OH})_2$.

with the results of the titration experiments which show that $\text{Ca}(\text{OH})_2$ and $\text{Ba}(\text{OH})_2$ combine with gelatin in equivalent proportions and that, hence, the cation in combination with the gelatin is in this case bivalent.²

The results show clearly that the Hofmeister series are not the correct expression of the relative effect of ions on the swelling of gelatin, and that it is not true that chlorides, bromides, and nitrates have "hydrating," and acetates, tartrates, citrates, and

¹ LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 247, 1920-21.

² The method used in these experiments with alkali was not the same as that used in the experiments with acids, and hence cannot be used to compare the relative effect of acid with that of alkali.

phosphates "dehydrating," effects. If the pH of the gel is taken into consideration, it is found that for the same pH of the gel the effect on swelling is the same for all acids with monovalent anion, while the swelling is considerably less when the anion of the acid is divalent. Hence, only the valency, and not the chemical nature of the ion in combination with gelatin, affects the degree of swelling. We shall see in the second part that, according to Procter and Wilson, acids influence swelling by changing the osmotic pressure inside the gel through the establishment of a Donnan equilibrium, and that the force which resists and limits the swelling is the cohesion between the protein molecules of the gel. It is obvious that the anion of an acid may influence both the Donnan equilibrium between the solute inside and outside the jelly as well as the force of cohesion between the particles. This latter influence is almost negligible in the case of the swelling of the gel of gelatin, but it is striking in the case of solid particles of casein. Granules of casein swell and are soluble in HCl but not in trichloroacetic acid. Casein is more soluble in HCl than in HNO_3 . This influence of anions on solubility and cohesion must be strictly separated from the influence of acids on colloidal behavior, since the phenomenon of solubility is a general phenomenon of both crystalloids and colloids, and not a special colloidal phenomenon.

The older authors, who came to the conclusion that the Hofmeister series hold for this effect of acids on swelling, made the mistake of not measuring the pH of the gel, and hence mistook the effect of differences in the pH of the gel for differences in the effect of the chemical nature of the anion of the acid. This is illustrated by a recent paper¹ from the physico-chemical laboratory in Leipsic, in which the author intends to show that different acids affect the swelling of gelatin not according to the valency rule but according to the nature of the anion of the acid—in other words, in agreement with the Hofmeister series. Kuhn calculates the hydrogen ion concentration of his protein gels from Kohlrausch's tables as if the pH were the same in the presence of a protein as in water free from protein, which is contrary to fact. Moreover, Kuhn entirely overlooked the fact that, on account of the Donnan equilibrium, the pH inside the gel is different from

¹ KUHN, A., *Kolloidchem. Beihefte*, vol. 14, p. 148, 1921-22.

that outside. It is, however, necessary to measure the pH of the gel at equilibrium for the purpose of comparison. Further, Kuhn overlooked the fact that it is necessary to bring the gelatin to the isoelectric point before the acid is added, otherwise the quantities of acid added cannot be used for any calculation of the hydrogen ion concentration of the protein solution; and, finally, it must be insisted upon that the activity coefficients (*i.e.*, the pH), and not the conductivity ratios of acids, are the correct expression of the relative efficiency of acids. It is hardly necessary to point out that from experiments as uncritical as those of Kuhn no conclusion can be drawn. Similar errors had been made by the other experimenters who are believed to have confirmed the Hofmeister series.

(C) VISCOSITY

The valency rule, which permits us to predict the relative osmotic pressure of solutions of protein, holds also in the case of viscosity of gelatin solutions.

We will begin with experiments on the influence of gelatin on the viscosity of water.¹ A 4 per cent stock solution of isoelectric gelatin was prepared, and some of the stock solution was heated to 45°C. and made up to a 1.6 per cent solution in quantity sufficient for a day's experiments. This 1.6 per cent solution was kept during the day at 24°C. To 50 c.c. of this solution was added the desired acid or alkali in sufficient quantity, and the volume raised to 100 c.c. by the addition of enough distilled water. The 0.8 per cent solution was rapidly brought to a temperature of 45°, kept there for 1 minute, and was then rapidly cooled to 24°C. The solution was stirred constantly during the heating and cooling. The viscosity was measured immediately after the solution was cooled to 24°C. The measurements were all made at 24°C. by using the time of outflow through a viscometer. The time of outflow of distilled water through the Ostwald viscometer used was exactly 1 minute at 24°C. Each measurement of viscosity was repeated with the same gelatin solution and the beginning and the end of a series consisted in the measurement of viscosity of isoelectric gelatin. These latter measurements agreed in all experiments within 1

¹ LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 85, 1920-21.

second, varying only between 80 and 81 seconds, thus guaranteeing the reproducible character of the experiment.

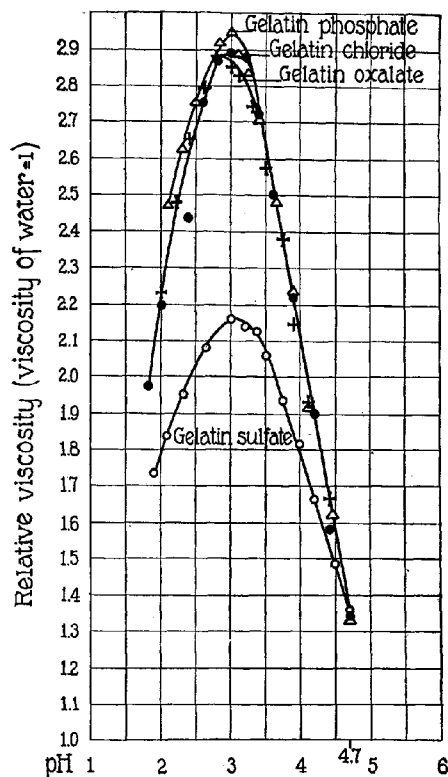


FIG. 36.—Curves representing relative viscosity of 0.8 per cent solution of originally isoelectric gelatin brought to different pH. The curves for relative viscosity of gelatin chloride, phosphate, and oxalate are practically identical. Relative viscosity is given as time of outflow of gelatin solution divided by time of outflow of water through viscometer at 24°C.

The results can be given briefly. Figure 36 gives the curves for the relative viscosity of 0.8 per cent solutions of gelatin

chloride, sulphate, oxalate, and phosphate. The abscissæ are the pH of the gelatin solutions, the ordinates the ratio of the time of outflow of the gelatin solutions divided by the time of outflow

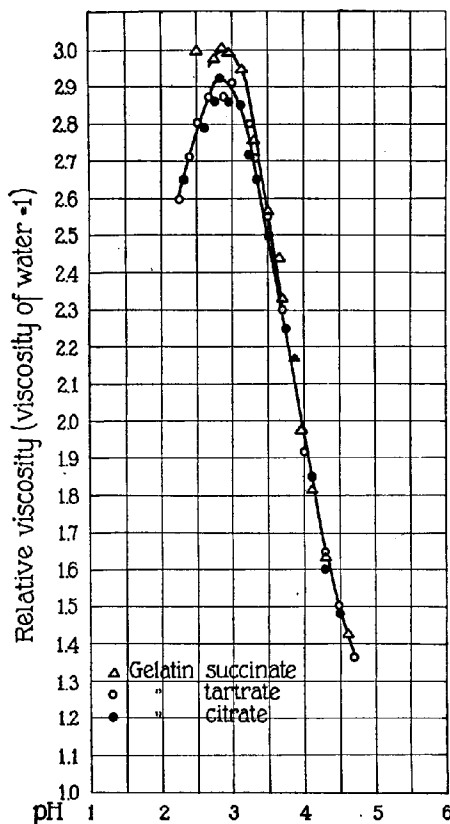


FIG. 37.—Curves representing relative viscosity of gelatin succinate, tartrate, and citrate. The curves are practically identical with those for the viscosity of gelatin chloride and phosphate.

of pure water. For the sake of brevity this quotient will be called the relative viscosity of the gelatin solution. The curves

for the four acids all rise steeply from the isoelectric point with increasing hydrogen ion concentration until they reach a maximum at pH about 3.0 or slightly above. The curves then drop

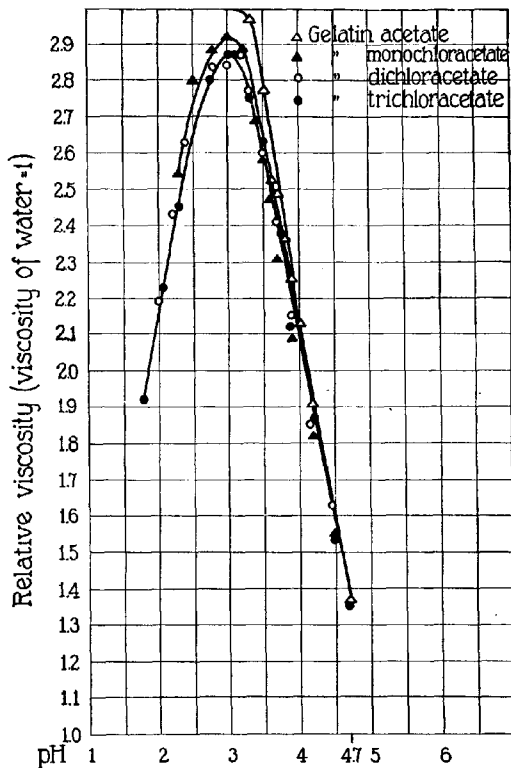


FIG. 38.—Curves representing relative viscosity of gelatin acetate, mono-, di-, and trichloracetate. Curves identical with those for gelatin chloride and phosphate.

again. The curves for the three salts, gelatin chloride, oxalate, and phosphate are practically identical; the curve for gelatin sulphate is considerably lower.

Figure 37 gives the curves for the viscosity of gelatin citrate, tartrate, and succinate. The three curves are practically identical and also almost identical with the curves for gelatin chloride and gelatin phosphate in Fig. 36.

Figure 38 gives the curves for the viscosity of 0.8 per cent solutions of originally isoelectric gelatin, to which acetic and mono-, di-, and trichloroacetic acids have been added. The curves are again identical with those for gelatin chloride, phosphate, etc., except that the viscosity curve for acetic acid is slightly higher than that of the rest. This anomalous behavior of acetic acid was also noticed in the case of the swelling, but not in the case of osmotic pressure. The influence of acid on the viscosity of gelatin is, in reality, an influence on the swelling of small aggregates of jelly in the gelatin solution, as will be shown in the second volume. This suggests that the excessive influence of high concentrations of acetic acid on viscosity may have the same source as that on swelling—being due to the large concentration of non-dissociated acid, possibly on the forces of cohesion of the gel.

The titration curves with alkalis have shown that Ca and Ba combine with proteins in equivalent proportions and we should hence expect that the viscosity curves for Ba and Ca proteinates would be lower than those for Li, Na, K, and NH_4 proteinates. This was found to be correct.

In experiments on the viscosity of casein solutions the limited degree of solubility of the salts of casein has to be considered. In the region from 4.7 to 3.0 or even a trifle below neither casein chloride nor casein phosphate is sufficiently soluble to permit the preparation of a 1 per cent solution, and in this region the influence of casein on the viscosity of water is therefore negligible. The curve representing the relative viscosity of 1 per cent casein chloride and phosphate solutions (as compared with that of pure water) rises sharply at pH 3.0. With a further increase of the hydrogen ion concentration the curve falls steadily, as it did in the case of the curve for gelatin. This indicates that the maximum for the influence of casein chloride on viscosity lies at pH equal to or greater than 3.0. The curve for the influence of casein phosphate on viscosity coincides with the curve for casein chloride.

The difference between the viscosity curve of Na caseinate and Ba caseinate (Fig. 39) is also similar to that of the corresponding gelatin salts.

It was found that acids and alkalis influence the viscosity of crystalline egg albumin only little if the temperature and concentration of the albumin are not too high. This is of great theoretical importance as will be shown in a later chapter.

All these experiments can be summarized as follows. When acids are added to a solution or a gel of isoelectric protein, the

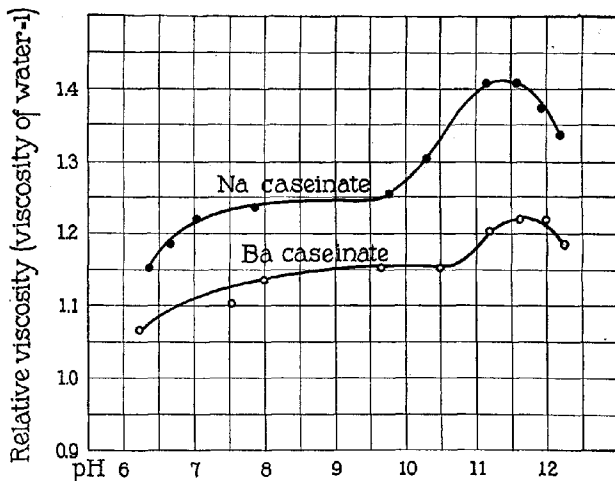


Fig. 39.—Curves representing relative viscosity of Na and Ba caseinate for different pH.

osmotic pressure, and, in the case of some proteins, the viscosity of the protein solution, and the swelling of gels are affected in a similar way. The values of these three properties rise until a maximum is reached which is followed by a drop if more acid is added. All the acids with monovalent anion act quantitatively alike when their effect is compared at the same pH of the protein solution or the protein gel; and all the acids with bivalent anion act alike, but the effect of the latter acids is considerably lower than that of the former. It follows, therefore, that only the valency but

not the chemical nature of the anion of the acid has an effect on these three properties of proteins. This contradicts the so-called Hofmeister anion series, which were based on a methodical error, namely, the failure of the former authors to measure the hydrogen ion concentration of their protein solutions or gels and to compare the effect of acids at the same pH of the protein solution or protein gel. This valency rule is of the utmost importance for the theory of colloidal behavior, since it leads to the conclusion that the colloidal behavior must be due to the Donnan equilibrium, which, being an electrostatic equilibrium, depends, therefore, on the valency but not on the chemical nature of the anion. What has been stated for the action of acids is also true for the action of alkalis on isoelectric protein, except that the effect of different alkalis depends on the valency of the cation of the alkalis.

CHAPTER VIII

THE ACTION OF NEUTRAL SALTS ON THE PHYSICAL PROPERTIES OF PROTEINS

1. THE DIFFERENCE IN THE EFFECT OF ACIDS, ALKALIES, AND SALTS ON PROTEINS

The most striking proof for the alleged existence of specific ion effects on proteins (aside from those due to valency of the ion) seemed to have been furnished by experiments on the influence of neutral salts on the osmotic pressure, the swelling, and the viscosity of protein solutions.

It has been noticed by a number of authors that the influence of neutral salts on the physical properties of proteins differs from that of acids and bases, and various attempts have been made to find an accurate expression for this difference. Some hold that neutral salts form "adsorption compounds" with "electrically neutral," *i.e.*, non-ionized, protein molecules, in which both ions of the salt were believed to be simultaneously adsorbed by the "neutral" protein molecule.¹ This idea is no longer tenable for salt solutions (as long as the concentration of the salt is not too high), since the experiments with powdered gelatin discussed in Chap. II have shown that only one (or practically only one) of the two ions of a neutral salt can combine at one time with a protein. At the isoelectric point, *i.e.*, at pH 4.7, gelatin can practically combine with neither ion of a neutral salt; at a pH > 4.7 only the metal ion of the neutral salt can combine with the gelatin, forming metal gelatinates; at a pH < 4.7 only the anion of the neutral salt is capable of combining with the protein, forming gelatin-acid salts.

Lillie has made the statement that, while acids and alkalies increase, salts depress the osmotic pressure of gelatin.² This statement, while it was the expression of facts actually observed

¹ PAULI, W., *Fortschritte naturwiss. Forschung*, vol. 4, p. 223, 1912.

² LILLIE, R. S., *Am. J. Physiol.*, vol. 20, p. 127, 1907-08.

by Lillie, is not entirely correct, because the influence of the hydrogen ion concentration of the gelatin solution was not taken into consideration. It will be shown that if acid is added to a gelatin-acid solution of a pH of 2.5 or below, the effect is the same as when we add a neutral salt, namely, a diminution of the osmotic pressure of the solution; and that when alkali, *e.g.*, KOH, is added to a solution of a metal gelatinate of pH 11.0 or above, the effect is also a similar depression of the osmotic pressure to that caused by the addition of KCl.

A depression is also noticed when some acid is added to a solution of metal gelatinate or when some alkali is added to gelatin-acid salts, since in both cases the gelatin is brought nearer to the isoelectric point.

It is also incorrect to speak of an antagonism between the effects of acids and salts, since the facts mentioned show that there is also an antagonism between little and much acid; thus, if the pH of a gelatin-acid salt is 3.0, a further addition of the same acid depresses the osmotic pressure or viscosity. The question then arises: What is the correct expression of the facts in the case?

The answer seems to be as follows: Suppose the pH be that of the isoelectric point of a protein and HCl be added. In this case the more acid is added the more non-ionogenic protein is transformed into salt. This salt formation raises the osmotic pressure, swelling, and viscosity of the protein. This agrees with the views of Laqueur and Sackur, and of Pauli, although the reason given by these authors for the effect of ionization of the protein on the colloidal properties is not correct, as will be shown in the second part of this book. At the same time the anion of the acid has an opposite, namely, a depressing effect. The addition of acid to isoelectric protein has therefore two opposite effects on the osmotic pressure, viscosity, and swelling of protein, namely, first, an augmenting effect due to increasing protein-salt formation with increasing hydrogen ion concentration, and second, a depressing effect due to the anion, in our example Cl. At first, the augmenting effect increases more rapidly than the depressing effect because, when little acid is added to isoelectric protein, protein salt is formed. When, however, the pH of the protein solution approaches the value 3.0, the augmenting influence due

to the formation of new gelatin chloride grows less rapidly with a further decrease in pH than does the depressing effect of the anion, and, hence, when the amount of acid added increases still further, the depressing effect of the Cl ion prevails over the augmenting effect of the H ion.

When an alkali, *e.g.*, NaOH, is added to an isoelectric protein, *e.g.*, gelatin, at first more of the non-ionogenic protein is transformed into metal proteinate, *e.g.*, Na gelatinate; and this raises the osmotic pressure, viscosity, and swelling rapidly by causing an increase in the concentration of ionized protein for a reason which will be given later. The cation of the alkali, the Na ion, has a depressing effect on these properties, and this depressing effect begins to be visible when the pH exceeds a certain value. After this, with a further addition of alkali, the depressing action of the cation (*e.g.*, of Na) increases more rapidly than the augmenting action of the OH ion.

When, however, a salt is added to a solution of a protein or to a protein gel, no augmenting effect on the osmotic pressure or viscosity of protein solutions or on the swelling of gels is possible, since any augmenting effect on these properties can only be caused by an increase in the ionization of the protein. It has been proved by experiments on cataphoresis of protein particles,¹ as well as on osmosis through gelatin films,² that when a neutral salt, like LiCl, NaCl, KCl, MgCl₂, CaCl₂, SrCl₂, BaCl₂, Na₂SO₄, is added to isoelectric protein, no protein salt and no protein ions are formed, provided the solution is kept at the pH of the isoelectric point. This conclusion was also confirmed by experiments on membrane potentials which will be mentioned in the second part of this volume. If it is true that the augmenting effect of acid and alkali on osmotic pressure and viscosity of protein solutions and on the swelling of protein gels is due to the formation of protein ions, it was to be expected that the addition of salts like NaCl, Na₂SO₄, or CaCl₂ to solutions of isoelectric gelatin or other proteins should not raise the osmotic pressure of these solutions, and this was found to be correct.³ Only salts with trivalent or tetravalent ions cause a slight rise in the osmotic

¹ LOEB, J., *J. Gen. Physiol.*, vol. 5, p. 395, 1922-23.

² LOEB, J., *J. Gen. Physiol.*, vol. 4, p. 463, 1921-22.

³ LOEB, J., *J. Gen. Physiol.*, vol. 4, p. 741, 1921-22.

pressure of solutions of isoelectric gelatin, but in that case it is probable that some change in the hydrogen ion concentration occurs.¹

On the other hand, if NaCl or CaCl₂, etc. are added to a solution of gelatin chloride of pH 3.0, where the osmotic pressure is comparatively high, the salt must have a depressing effect because the concentration of the anion of the protein salt is increased, and this depression must be the same as if the concentration of anions had been increased by the addition of more HCl. When NaCl or CaCl₂ is added to sodium gelatinate of pH 11.0 or 12.0, where the osmotic pressure is high, the increase in Na ions by the salt will have the same depressing effect as the increase in the concentration of Na ions by adding NaOH. The correctness of these deductions is supported by the following experiments.

An approximately 1.6 per cent solution of isoelectric gelatin was prepared and brought to a pH of 4.0. The solution was made 0.8 per cent in regard to the originally isoelectric gelatin by adding to 50 c.c. of the 1.6 per cent solution either 50 c.c. of H₂O or of a salt solution, *e.g.*, NaCl, of different molecular concentration, from $m/8,192$ to 1 M, taking care that the hydrogen ion concentration remained the same. The time of outflow through a viscometer was determined in the way described in Chap. V, and the ratios of the time of outflow to that of water were plotted as ordinates over the pCl as abscissæ (lower curve, Fig. 40). We will designate this value as relative viscosity. The addition of the NaCl causes only a drop, and no rise in the curve.

If, however, the 1.6 per cent gelatin solution of pH 4.0 is mixed with various concentrations of HCl (upper curve, Fig. 40), instead of with NaCl, at first a rise occurs which is followed by a drop when the concentration of the Cl ion is a little above $n/1,000$. In Fig. 40 the drop appears at a concentration of about $n/256$ HCl, but the reader must remember that, because part of the acid combined with the gelatin, the pH of the solution was about 3.0. In other words, while the addition of H ions increases the viscosity of a solution of gelatin chloride of pH 4.0, on account of the increase in the formation of gelatin chloride, the addition of Na ions does not have such an effect, but the Cl ion depresses

¹ LOEB, J., *J. Gen. Physiol.*, vol. 5, p. 505, 1922-23.

the viscosity in both cases, no matter whether NaCl or HCl is added to the gelatin solution; and the depressing action of the Cl ion increases with its concentration. Moreover, the

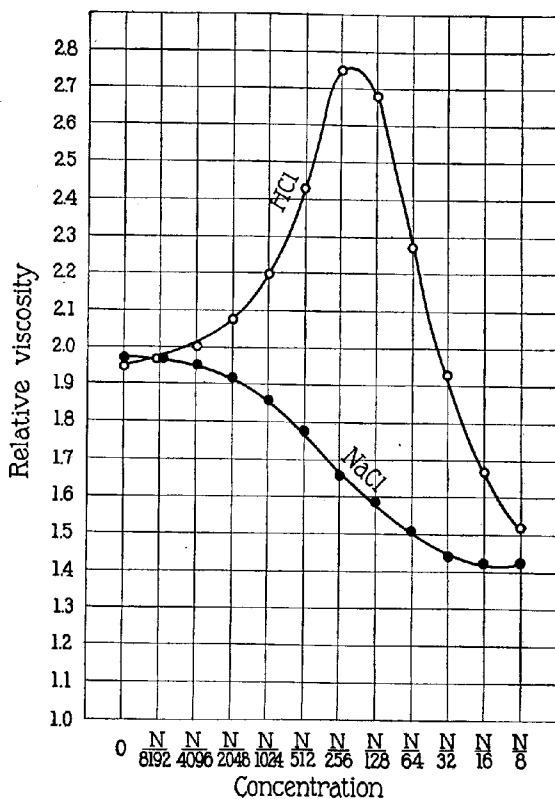


FIG. 40.—Difference in the effect of different concentrations of NaCl and of HCl on the relative viscosity of an 0.8 per cent solution of gelatin chloride of pH 4.0. In the case of NaCl we observe only the depressing effect of the Cl ion; in the case of HCl we notice an augmenting effect of the H ion and a depressing effect of the Cl ion, the latter prevailing as soon as the concentration of acid added is $> N/256$.

increase of the viscosity by the H ions stops as soon as the pH of the solution reaches about 3.0.

When the same experiment is repeated with a gelatin solution of pH 3.0, the addition of NaCl immediately causes a drop also (Fig. 41), while the addition of HCl no longer causes a rise but only a drop, though the drop commences a little later than in the case of NaCl.

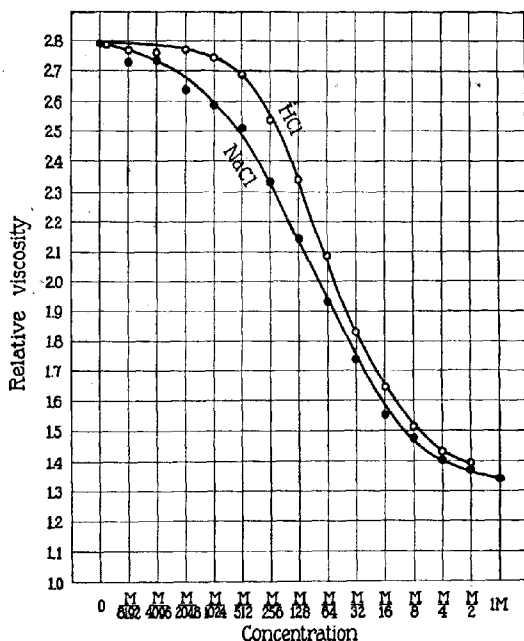


FIG. 41.—The relative viscosity of 0.8 per cent solution of gelatin chloride of pH 3.0 is depressed almost equally by the Cl ion of HCl as of NaCl. The augmenting effect of the H ion in the case of HCl is no longer noticeable.

When, however, the same experiment is made with a gelatin solution of pH 2.5 (Fig. 42), an immediate drop is noticed upon the addition of HCl as well as upon the addition of NaCl, and the curve for HCl coincides practically with that for NaCl as our theory demands, since at this pH all the gelatin exists in the form of gelatin chloride, as shown by Fig. 9 in Chap. IV. Hence,

the further addition of HCl to gelatin chloride of pH 2.5 cannot increase the concentration of ionized gelatin.

The same difference as between the action of acids and salts exists between the action of alkalies and salts. When enough KOH

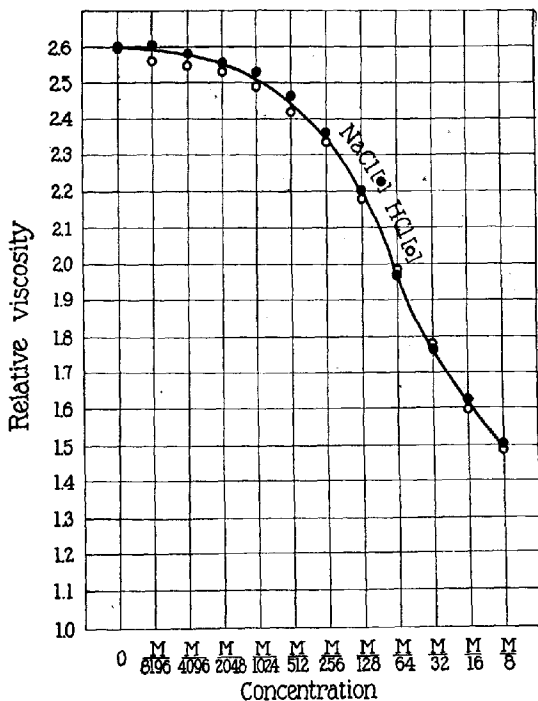


FIG. 42.—When the gelatin solution has a pH of 2.5, HCl and NaCl depress the relative viscosity of the gelatin solution to the same degree.

is added to isoelectric gelatin to transform all the gelatin present into K gelatinate, the further addition of KOH should act quantitatively as the addition of KCl. At pH 12.0 all the isoelectric gelatin is transformed into K gelatinate (when the alkali added is KOH). Figure 43 shows that the addition of equal molar con-

centrations of KOH and of KCl to a solution of K gelatinate of pH 12.0 has quantitatively the same effect on the viscosity of the solution.

The depressing effect of neutral salts on the physical properties of proteins is, therefore, the same phenomenon as the drop in the curves of these properties when too much acid or too much alkali has been added. It is due to the fact that in all cases that ion which has the opposite sign of charge to that of the protein ion depresses the osmotic pressure, swelling, and viscosity of proteins.

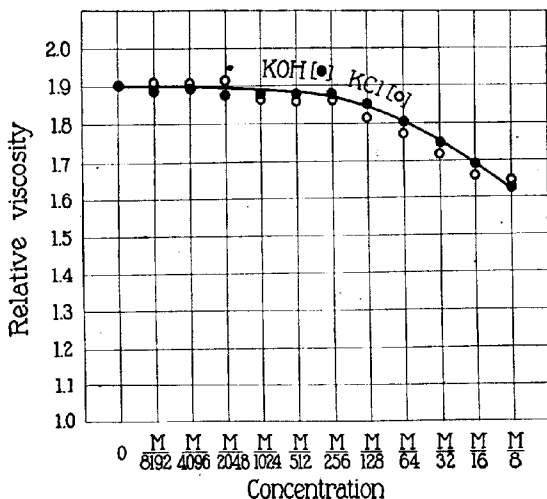


FIG. 43.—The depressing effect of KOH and KCl on Na gelatinate of pH 12.0 is practically the same.

2. VALENCY RULE AND THE ACTION OF SALTS ON PROTEINS

From what has been said it is clear that, if the pH of the protein solution is kept constant, only one of the ions of a neutral salt influences the colloidal properties of a protein, namely, that ion which has the opposite sign of charge to the protein ion; and this influence is of a depressing character. We will now show that for osmotic pressure, swelling, and viscosity this effect depends only upon the valency of the depressing ion and that different ions of the same valency have the same depressing effect.

This is contrary to the view generally expressed in the colloidal literature, according to which both ions of a salt influence the osmotic pressure, viscosity, and swelling of proteins and this influence is said to vary not only with the valency but also with the chemical nature of the ion. The relative efficiency of different anions and cations is expressed in the so-called Hofmeister ion series. Those who believe in the validity of the Hofmeister series for the action of salts on the three above-mentioned properties of proteins arrange the effect of salts on the swelling of gelatin in the following way:

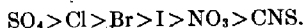
SO_4 , tartrate, citrate < acetate < Cl < Br , NO_3 < I < CNS , where the swelling is said to be a maximum in CNS and a minimum in SO_4 .

Above a certain concentration, the sulphates, tartrates, and citrates cause a shrinkage of the gel of gelatin and acetate acts in the same sense but less strongly; while the other anions cause an increasing swelling of the gel of the same concentration.¹

As far as the actions of the cations of a salt are concerned, Höber makes the following statement:

The differences in the effects of cations are less marked; it might be possible to propose the series Li < Na < K , NH_4 ; then follow the alkali earths with Mg in a position between.

Höber states that these anion and cation series are correct at a neutral reaction. Since the isoelectric point of gelatin is at pH 4.7, at neutral reaction, *i.e.*, pH near 7.0, gelatin exists as metal, the properties of which should not be affected at all by the anions of a salt and should be affected only by the valency of the cation of a salt, but not by any other properties of the cation. As far as osmotic pressure is concerned, Lillie's experiments are quoted by Höber, who states that salts depress the osmotic pressure of neutral solutions of egg albumin in the following order:



At neutral reaction of the albumin solution the anions of a salt should have no influence on the osmotic pressure of a protein

¹ HÖBER, R., "Physikalische Chemie der Zelle und der Gewebe," 5th ed. part 1, p. 267, Leipzig, 1922.

solution. Neither Lillie nor any of the other authors had measured the pH of their solutions or gels, and the Hofmeister ion series in osmotic pressure and swelling are the result of this methodical error, as a consequence of which these authors mistook effects due to variation of the pH for effects due to the nature of the anion. If we wish to study the effect of anions on the three physical properties of proteins—osmotic pressure, swelling, and viscosity—we must use protein-acid salts, the pH of which is, in the case of gelatin, less than 4.7, and we must make sure that the pH of the mixture of protein solution and salt or protein gel and salt remains constant. This can be done with the aid of solutions of a definite pH, which must be controlled with the hydrogen electrode, as will be shown in the following experiments.

3. OSMOTIC PRESSURE

For these experiments three stock solutions, all of pH 3.8, were prepared. First, solutions of gelatin chloride of pH 3.8 containing 2 gm. dry weight of originally isoelectric gelatin and 8 c.c. of 0.1 *N* HCl in a 100-c.c. solution; second, *m*/2 solutions of different salts brought to a pH of 3.8 by the addition of HCl; and third, distilled water brought also to the pH of 3.8 by the addition of HCl. By successive dilution of the *m*/2 salt solutions of pH 3.8 with distilled water of pH 3.8, series of salt solutions of different degrees of concentration, but all of pH 3.8, were prepared. Fifty cubic centimeters of the 2 per cent solutions of gelatin chloride of pH 3.8 and 50 c.c. of the salt solutions of pH 3.8 were then mixed and 1 per cent solutions of gelatin chloride in salt solutions of different concentrations but all of pH 3.8 were obtained.

In order to bring 100 c.c. of *m*/2 stock solution of different salts to a pH of 3.8 the solution had to contain different quantities of 0.1 *N* HCl as Table VIII shows. Attention is called to the enormous amount of HCl required to maintain pH 3.8 in the presence of *m*/2 acetate. Yet the older colloid chemists compared the effect of Na acetate with that of NaCl without correcting for the difference in pH.

Collodion bags containing the mixture of gelatin chloride and salt solution were dipped into beakers containing 350 c.c. of

TABLE VIII.—CUBIC CENTIMETERS OF 0.1 N HCl IN 100-C.C. M/2 SOLUTIONS OF SALT REQUIRED TO PRODUCE A pH OF 3.8

	Cubic centimeters of 0.1 N HCl required
NaCl.....	0.2
NaBr.....	0.6
NaI.....	0.3
NaNO ₃	0.3
NaCNS.....	0.7
Na acetate.....	425.0
Na ₂ SO ₄	1.0

water of pH 3.8. The method of the experiments was as previously described.

Figure 44 represents the effect of six different salts with monovalent anions, namely, NaCl, NaBr, NaI, NaNO₃, NaCNS, and Na acetate, and one salt with divalent anion, namely, Na₂SO₄, on the osmotic pressure of a gelatin chloride solution of pH 3.8. These salts were selected to find out whether the above-mentioned Hofmeister series are real or fictitious. The abscissæ are molar concentrations of the salts and the ordinates are the observed osmotic pressures in terms of the height of a column of water.

The striking result is that at the same pH the influence of the six salts with monovalent anion is exactly the same, since the values for the influence of all these six salts lie all on one curve. The variations in their value are the chance variations due to the limits of accuracy of the experiments. The osmotic pressure is a little over twice as high when the anion of the salt is monovalent than when it is divalent.

This shows that all the salts with monovalent anions have the same effect on the osmotic pressure of a gelatin chloride solution, when the pH is kept constant, and Lillie's anion series are based on error. Only the valency, but not the chemical nature of the anion of the salt, influences the osmotic pressure of the gelatin chloride solution. This statement is supported by experiments on the effect of salts, the anion of which forms weak dibasic acids, namely Na₂ succinate, Na₂ tartrate, and Na₂ oxalate. Since these

salts are buffer salts, enormous quantities of HCl had to be contained in 100 c.c. of $M/2$ solutions of such salts to produce a pH of 3.8.

Na ₂ oxalate.....	55.0 c.c. 0.1 N HCl
Na ₂ tartrate.....	150.0 c.c. 0.1 N HCl
Na ₂ succinate.....	430.0 c.c. 0.1 N HCl

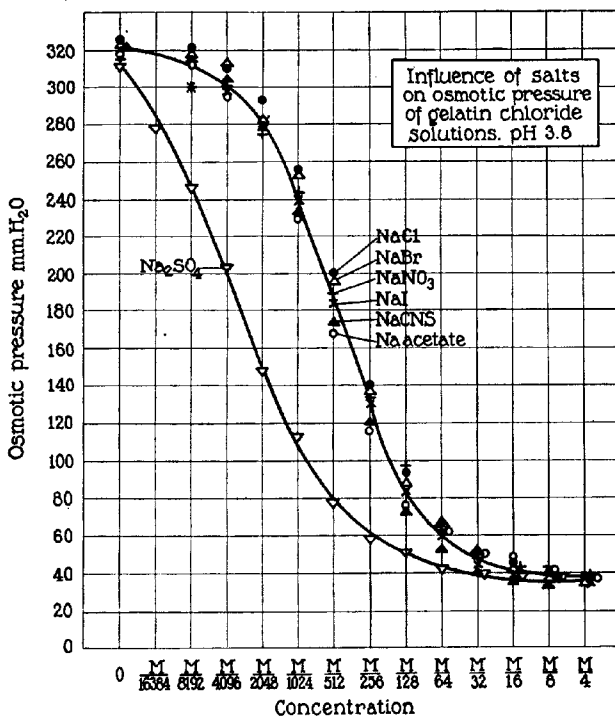


FIG. 44.—All salts with monovalent anions depress the osmotic pressure of gelatin chloride solutions of pH 3.8 to the same extent (within the limits of experimental accuracy). Na₂SO₄ depresses considerably more.

In the mixtures of HCl and these salts, weak dibasic acids are formed, the latter dissociating chiefly, though not exclusively, as monobasic acids at pH 3.8. By adding such buffer salts to a

solution of gelatin chloride of pH 3.8, and maintaining the pH by adding HCl, part of the bivalent anions of the salts are replaced by monovalent anions. Hence, the depressing effect of the three above-mentioned salts on the osmotic pressure of a gelatin chloride solution of pH 3.8 should lie between that of NaCl and that of Na_2SO_4 , but that of Na_2 oxalate or Na_2 tartrate

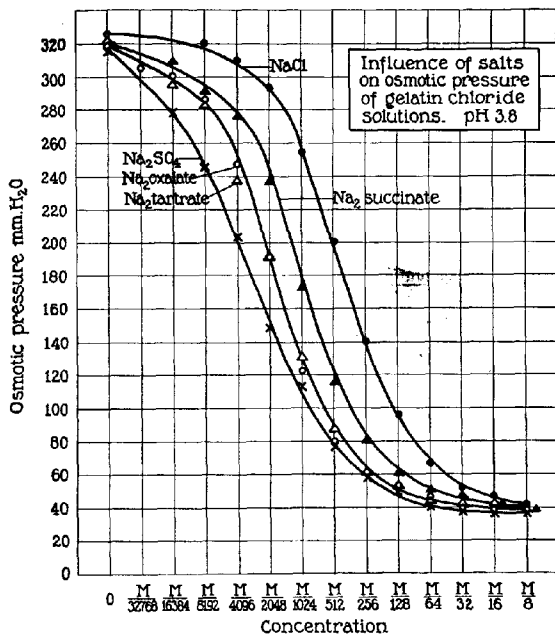


FIG. 45.—Depressing effect of weak dibasic and tribasic salts on osmotic pressure of gelatin chloride solutions of pH 3.8.

closer to that of Na_2SO_4 than that of sodium succinate. Figure 45 shows that this is correct.

The older authors ignored the pH and compared the effect of Na_2 tartrate with that of Na_2SO_4 at an entirely different pH. When the pH of the solution is kept constant, the influence of salts on the osmotic pressure of solutions of gelatin chloride is determined only by the valency of the anion of the salt, but not

by its chemical nature. The Hofmeister anion series for osmotic pressure effects are purely fictitious and the result of a methodical error.

The same can be shown to be the case for the so-called cation series referred to. According to the writer's view, the cations

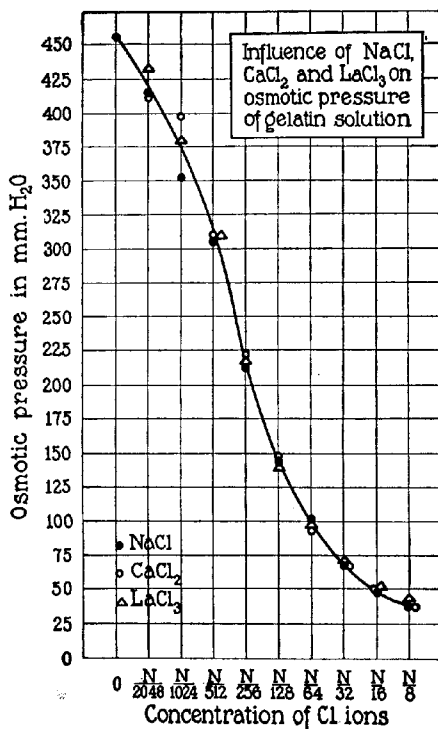


FIG. 46.—Influence NaCl, CaCl₂, and LaCl₃ on osmotic pressure of a 1 per cent solution of gelatin chloride of pH 3.0. Ordinates are osmotic pressures in millimeters H₂O, abscissae the concentrations of Cl ions of the salts. The depressing effect is the same for the three salts, proving that only the anion influences the osmotic pressure in this case.

should have no effect on the osmotic pressure of protein solutions on the acid side of the isoelectric point, and this is shown to be true in Fig. 46. In this experiment a pH of 3.0 of the gelatin

chloride solution and the stock solutions of salts and H_2O were chosen. Figure 46 gives the depressing effect of $NaCl$, $CaCl_2$, and $LaCl_3$ on the osmotic pressure of a 1 per cent solution of gelatin chloride of pH 3.0. The ordinates are the osmotic pressures after 18 hours at $24^\circ C.$, while the abscissæ are the concentrations of the Cl ions of the three salts. The depressing effect on the osmotic pressure is the same for equal Cl concentrations of the three salts, proving that only the anion of the salt influences the osmotic pressure of a gelatin chloride solution of pH 3.0 and that the cation has no influence whatever. The same fact was shown to be true in experiments of Hitchcock on the influence of these salts on the osmotic pressure of edestin chloride solutions.¹

We can, therefore, state that the cation as well as the anion series are fictitious as far as the influence of salts on the osmotic pressure of protein solutions is concerned and that the Hofmeister series in this case have to be replaced by the valency rule.

4. SWELLING

The general method described for measuring the influence of acids on swelling was also used for measuring the influence of salts on swelling. It was intended to use gels which at equilibrium had a pH of 3.8. It had been found in previous experiments that 1 gm. of powdered isoelectric gelatin has at equilibrium a pH of 3.82 when it is put into 150 c.c. of water containing 4.5 c.c. 0.1 N HCl .

The following stock material was prepared: first, doses of wet powdered isoelectric gelatin of 8 gm. each containing about 1 gm. dry weight of isoelectric gelatin; second, a stock solution of HCl containing 3 c.c. of 0.1 N HCl per 100 c.c. H_2O (since the isoelectric gel of gelatin had in such a solution at equilibrium a pH of 3.82); and third, $m/2$ solutions of various salts made up with HCl to the same pH as the stock solution of HCl . The stock solution of HCl alone, without gel or salt, was used for the dilutions of the $m/2$ salt solutions.

Doses of 8 gm. of the wet isoelectric gelatin (as described in the preceding chapter) were added to 150 c.c. of various salt solutions made up as described, and allowed to stand for 2 hours in the

¹ HITCHCOCK, D. I., *J. Gen. Physiol.*, vol. 4, p. 597, 1921-22.

solutions at 15°C. The swelling of the gelatin was then measured by weight, as described in the preceding chapter.

In the figures the abscissæ are the concentrations of salts and the ordinates are the weights. Without salts the weight varied generally at the end of the experiment around 27 gm., while it was depressed by the highest concentrations of salts used to about 10 gm.

The first fact to be ascertained was whether or not only the valency of the anion of the salt is of influence, or whether the anion series generally quoted in colloidal literature are valid,

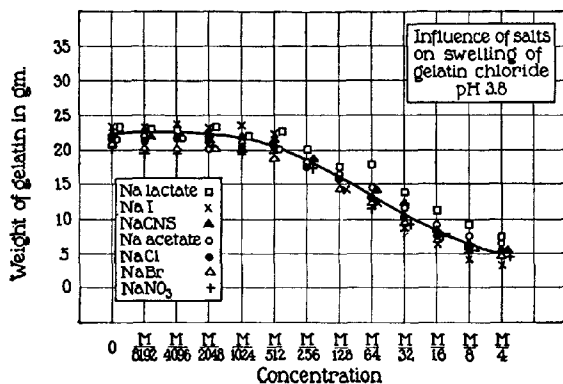


FIG. 47.—All salts with monovalent anions depress the swelling of a gelatin chloride gel to the same extent (within the limits of experimental accuracy) at pH 3.8.

according to which the swelling is a maximum in NaCNS, and a minimum in Na acetate (leaving the divalent anions out of consideration for the present).

Seven salts with monovalent anions were tried, namely, NaCl, NaBr, NaI, NaNO₃, NaCNS, Na acetate, and Na lactate. The results are given in Fig. 47. It is obvious that the values of the effects of all these seven salts lie on one curve and that the variations are essentially the chance variations due to the limits of experimental accuracy. This is proved by the fact that the same variations are observed when the concentration of salt is zero, *i.e.*, when no salt is added. There is not the slightest indication

of a Hofmeister anion series. Slight influences of the salts on the cohesion of the gel of gelatin may exist, but they are too small to play much of a rôle.

While salts with monovalent anions have the same depressing effect for the same concentration of anions, salts with bivalent anions have a much greater depressing effect on swelling than salts with monovalent anions. This is illustrated in Fig. 48, showing the difference in the effect of equal molar concentrations of NaCl and Na₂SO₄ on swelling. NaCl does not depress swelling in concentrations of M/1,024 or below and the depressing effect of NaCl on the swelling of gelatin chloride of pH 3.8

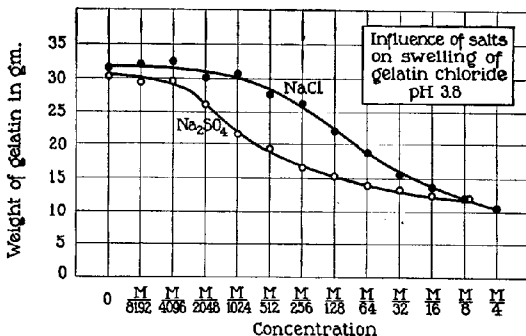


FIG. 48.—Na₂SO₄ depresses the swelling of a gelatin chloride gel considerably more than NaCl.

commences to be noticeable at a concentration of M/512. This is true for all salts with monovalent anions, as Fig. 48 shows. Na₂SO₄ begins, however, to depress at a concentration between M/4,000 and M/2,000, and the curve for the SO₄ effect drops much more rapidly to the minimum than the curve for the Cl effect.

It may be well to call attention here to the fact that it requires relatively high concentrations of salt to influence the physical properties of protein solutions or gels, M/512 or more for NaCl, and M/4,000 or more for Na₂SO₄. This should set at rest the remarks not substantiated by fact that the minutest traces of salts have already an influence on the physical properties of proteins.

It was to be expected that the influence of LiCl , NaCl , KCl , CaCl_2 , and LaCl_3 should be the same on the swelling of a gelatin gel at the same concentration of Cl ions of the salt, provided the pH is kept constant. Figure 49 shows that this expectation is correct. It is obvious that the effects of these five salts on swelling lie all on one curve, proving that the effect of salts on swelling of gelatin chloride is determined only by the anion of the salt and that the cation of the salt has no effect on the swelling of a gel of gelatin chloride. This eliminates the so-called cation series in this case.

Summarizing all these results, we may say that only the anion of a salt influences the swelling of a gel of gelatin chloride, and

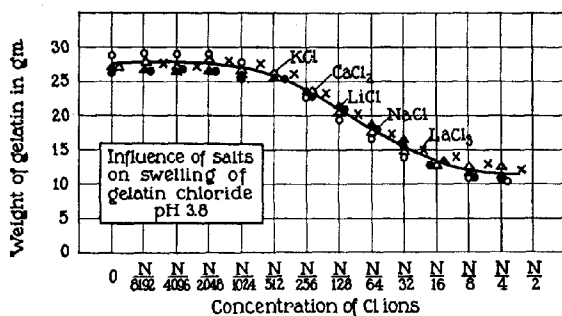


FIG. 49.—All chlorides depress the swelling of a gelatin chloride gel of pH 3.8 to the same extent at the same concentration of Cl ions.

only the valency, but not the chemical nature of the anion, has an effect on this property, provided the pH of the gel is kept constant.

5. VISCOSITY

What has been said for the effects of salts on the osmotic pressure and the swelling of gelatin holds also for the effect on viscosity. In these experiments the solution contained about 1.6 gm. dry weight of originally isoelectric gelatin in 100 c.c. The solution was brought to a pH of 3.0 or 3.3 by the addition of HCl . Fifty cubic centimeters of this solution was mixed with 50 c.c. of solution of a salt also of pH 3.0. These salt solutions were

made up from $m/2$ stock solutions brought to a pH of 3.0 and diluted with water of pH 3.0. Before measuring the viscosity in the viscometer the solution was heated rapidly to about 45°C . and then cooled rapidly to 24°C . and the time of outflow through the viscometer was measured, as described in the preceding chapter.

Figure 50 shows that under such conditions NaCl and Na acetate have an equal effect on the relative viscosity of a gelatin chloride solution; 1.6 per cent solutions of gelatin acetate of pH 3.3 and of gelatin chloride of pH 3.3 were made up separately and the viscosities of these solutions were tested in the way described and found to be identical. Fifty cubic centimeters of the solution of 1.6 per cent gelatin acetate of pH 3.3 was diluted with 50 c.c. of a Na acetate solution of pH 3.3; and 50 c.c. of the 1.6 per cent solution of gelatin chloride of pH 3.3 was diluted with 50 c.c. of NaCl solution of pH 3.3. The Na acetate solution of pH 3.3 was obtained by making up a $m/16$ solution of Na acetate in $1\frac{1}{2}$ M acetic acid and the various degrees of dilution of this $m/16$ Na acetate solution of pH 3.3 were brought about by dilution with pure acetic acid of pH 3.3. The non-dissociated molecules of acetic acid have no more depressing influence on the physical properties of proteins than have the molecules of any non-electrolyte. Figure 50 gives the curve representing the depressing effect of Na acetate on gelatin acetate of pH 3.3, when the pH is kept constant.

The gelatin chloride solution of pH 3.3 was made up in different concentrations of NaCl and the depressing effect of NaCl on the viscosity of gelatin chloride is also plotted in Fig. 50. It is obvious from the figure that the depressing effects of Na acetate and NaCl are identical when the pH is kept constant and identical in both cases.

The same fact was confirmed in a somewhat different way. A 1.6 per cent solution of gelatin chloride of pH 3.0 was made up in various concentrations of Na acetate, also of pH 3.0. In order to prepare Na acetate solutions of pH 3.0, a solution of $m/4$ Na acetate was made up in $m/4$ HCl and the various dilutions required for the experiment were obtained by diluting the mixture with $m/1,000$ HCl.

The 1.6 per cent gelatin chloride solution of pH 3.0 was diluted with 50 c.c. of this mixture, so that the resulting 0.8 per cent

gelatin chloride solution of pH 3.0 contained various concentrations of Na acetate (or more correctly of NaCl and Na acetate). The curve representing the depressing effect of this salt is given

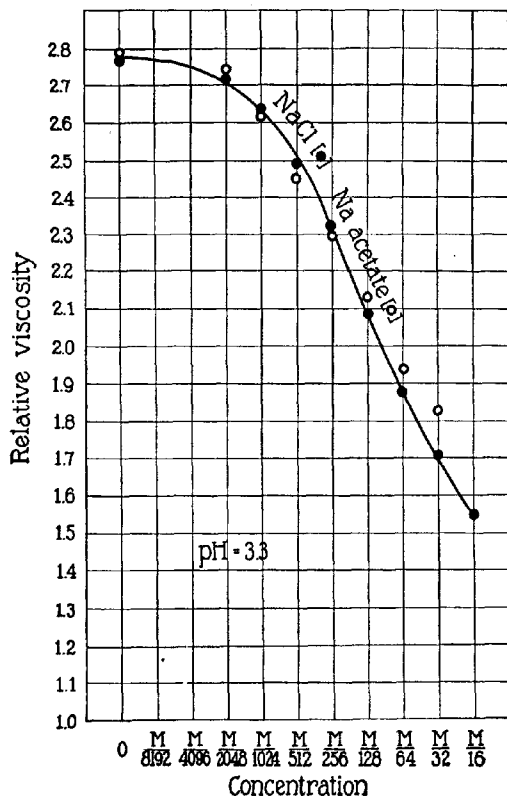


FIG. 50.—When the pH is kept equal, the depressing effect of equal concentrations of NaCl and Na acetate on the relative viscosity of an 0.8 per cent gelatin chloride or gelatin acetate solution of pH 3.3 is the same.

in Fig. 51, and is shown to be identical with the curve representing the depressing effect of the addition of NaCl to gelatin chloride of pH 3.0.

We can, therefore, state that sodium acetate has the same effect on the viscosity of gelatin chloride as the addition of any other salt with monovalent anion, and that the anomalous effect

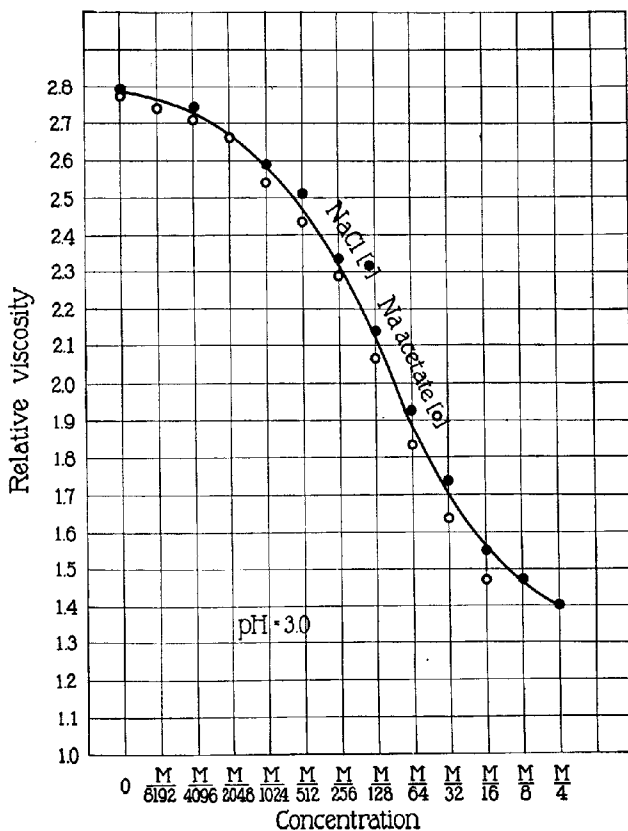


FIG. 51.—See legend of Fig. 50, except that the pH of gelatin solution is 3.0.

ascribed to the acetate anion in the colloidal literature is in reality due to the depression of the hydrogen ion concentration of the gelatin solution by the Na acetate, which is a buffer salt.

The depressing effect of a salt on the relative viscosity of a gelatin chloride solution increases rapidly with the valency of the anion, as is indicated in Fig. 52, which shows that the viscosity

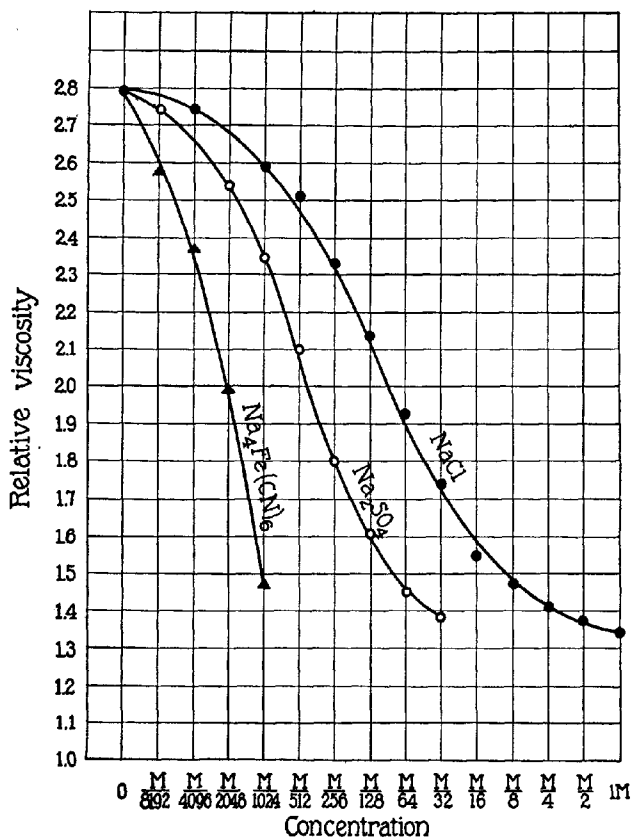


FIG. 52.—The relative depressing effect of equal molecular concentrations of NaCl , Na_2SO_4 , and $\text{Na}_4\text{Fe}(\text{CN})_6$ on the relative viscosity of a gelatin chloride solution of pH 3.0 is approximately as 1:4:16.

is depressed considerably more by Na_2SO_4 than by NaCl , and more by $\text{Na}_4\text{Fe}(\text{CN})_6$ than by Na_2SO_4 , though the curve for $\text{Na}_4\text{Fe}(\text{CN})_6$

cannot be considered as accurate on account of a possible pH error.

That the cations of salts have no effect on the viscosity is shown by the fact that NaCl , CaCl_2 , and LaCl_3 have the same

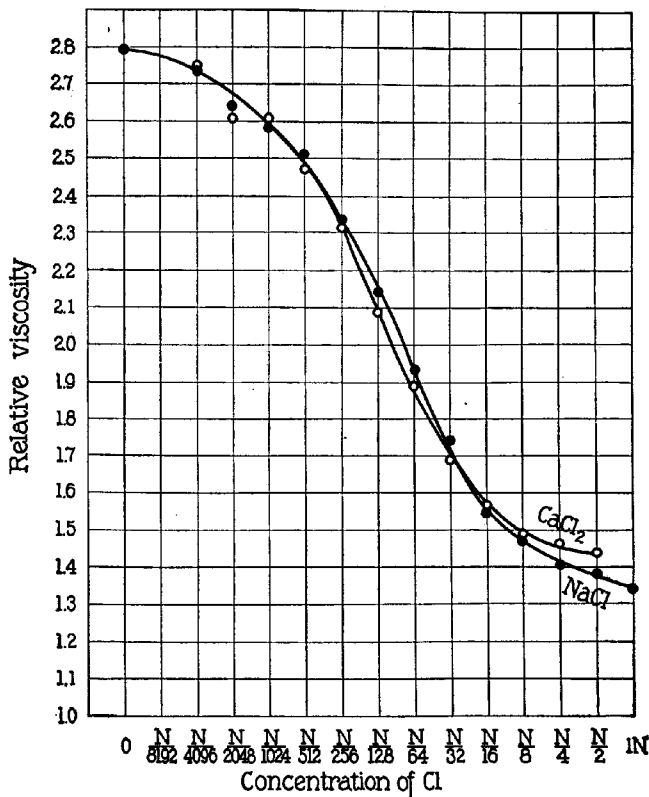


FIG. 53.—Showing that NaCl and CaCl₂ have the same depressing effect on the viscosity of gelatin chloride of pH = 3.0 when the concentration of Cl ions is the same.

effect on the viscosity of a gelatin chloride solution at the same pH when the concentrations of the Cl ions of the salts are identical.

This is illustrated by Fig. 53, giving the depressing effects of NaCl and CaCl_2 on the relative viscosity of a gelatin chloride solution of pH 3.0 containing about 0.8 gm. of originally isoelectric gelatin in a 100-c.c. solution. The abscissæ are the concentrations of the Cl ions of the salts. The effects of both salts lie on the same curve and the values for LaCl_3 were also on the same curve, though they are omitted in the diagram.

These figures may suffice to show that the valency rule holds also in the case of the effect of salts on the viscosity of gelatin solutions.

6. THE DEPRESSING EFFECT OF SALTS ON THE SWELLING OF GELS OF Na GELATINATE

The effect of salts on the three physical properties of gelatin mentioned, namely, osmotic pressure, viscosity, and swelling,

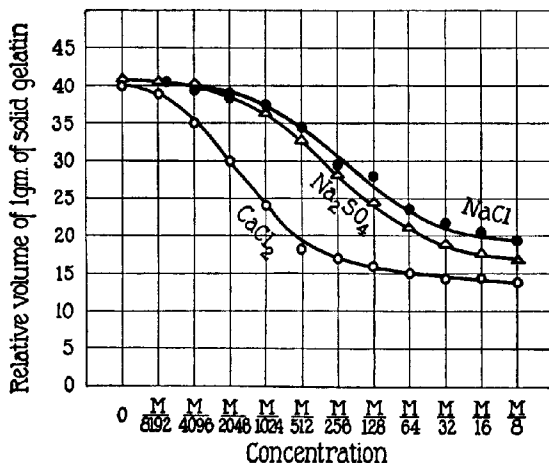


FIG. 54.—The depressing effect of neutral salts on the swelling of Na gelatinates of pH about 9.3 is due to the cation of the salt, the depressing effect of NaCl being half as great as that of Na_2SO_4 of equal molecular concentration of Na_2SO_4 , while that of CaCl_2 is considerably greater, because Ca is bivalent.

should be as follows when the gelatin is on the alkaline side of the isoelectric point. Only the cation of a salt should have a depressing effect, which should increase with the valency of the cation of

the salt, while the anion of the salt should have no effect. That this is true for the swelling of Na gelatinate (of pH of about 9.3) is shown in Fig. 54. The molecular concentration in which the swelling is depressed equally is about half as great for Na_2SO_4 as for NaCl (for molecular concentrations from $M/256$ to $M/32$), while it is about eight times as high for NaCl as for CaCl_2 , roughly proving that the cation is responsible for the depression. The pH of the gelatin was practically the same in all solutions.

All these data confirm our valency rule, whereby ions of the same valency and the same sign of charge have, in the same concentration, nearly the same depressing effect on osmotic pressure, swelling, and viscosity of proteins, while the depressing effect increases rapidly with the valency. The Hofmeister ion series are chiefly due to the failure to measure the influence of the salts on the hydrogen ion concentration of the gelatin solutions and gelatin gels.

We shall see in the second part of the book that the valency rule is a necessary consequence of the theory of colloidal behavior based on Donnan's theory of membrane equilibria.

7. THE ACTION OF NON-ELECTROLYTES

Previous authors had already observed that non-electrolytes, like cane sugar, have no effect on such physical properties of protein as osmotic pressure, viscosity, and swelling. Since in these older experiments the pH was not considered, and since this fact is of paramount importance, it seemed desirable to repeat the experiments. It was confirmed that non-electrolytes, like cane sugar, have no depressing effect on the osmotic pressure or the viscosity of gelatin solutions. Solutions of gelatin chloride of pH 3.4 containing 1 gm. of originally isoelectric gelatin in a 100-c.c. solution were made up in various concentrations of cane sugar, were rapidly heated to 45°C ., and rapidly cooled to 24°C . The time of outflow of the gelatin solutions through a viscometer was measured immediately. In addition, the time of outflow of the pure sugar solution was also determined at 24°C . The ratio of the time of outflow of the gelatin-cane sugar solution divided by the time of outflow of the pure cane sugar solution was thus determined. The results given in Table IX show that the ratio of viscosity of gelatin solution to viscosity of cane sugar solution is

not diminished by the addition of cane sugar; in fact it seems, if anything, slightly increased if the cane sugar concentration is above $m/8$.

Similar results were obtained in regard to osmotic pressure, as Table IX shows.

TABLE IX.—INFLUENCE OF THE ADDITION OF CANE SUGAR ON THE VISCOSITY AND OSMOTIC PRESSURE OF 1 PER CENT SOLUTIONS OF GELATIN CHLORIDE OF pH 3.4

	Concentration of cane sugar										
	0	$m/1,024$	$m/512$	$m/256$	$m/128$	$m/64$	$m/32$	$m/16$	$m/8$	$m/4$	$m/2$
Viscosity ratio.....	2.33	2.31	2.33	2.35	2.30	2.31	2.31	2.30	2.39	2.44	2.57
Osmotic pressure after 21 hours at 24°C. in milli- meters H ₂ O.....	434	390	380	405	408	400	407	432	397	401	395

This fact is one of the prerequisites for the validity of the theory of membrane equilibrium, since only ions contribute to the equilibrium conditions on opposite sides of the membrane.

A second prerequisite is that the addition of salts should have no influence on the viscosity, osmotic pressure, or P.D. of protein solutions at the isoelectric point. This prerequisite of the Donnan theory was also fulfilled, as has already been stated.

8. VALENCY RULE AND ADSORPTION HYPOTHESIS

It may be well to call attention to the fact that the refutation of the reality of the Hofmeister ion series for the influence of electrolytes on swelling, osmotic pressure, and viscosity of proteins affects the idea of an adsorption of ions by proteins. As long as the Hofmeister series for these properties were believed to be real, it was possible to cling to the belief that both ions of a salt might be adsorbed by a protein. This idea was made doubtful by the experiments described in Chap. II, which showed that the cations of a salt combine with a protein only on the alkaline side and that

the anions of a salt combine with a protein only on the acid side of the isoelectric point, but that the oppositely charged ions of a salt do not combine simultaneously at the same pH with a protein. This conclusion is corroborated by the experiments on the action of salts on the physical properties of proteins, such as osmotic pressure, swelling, and viscosity, as given in this chapter. On the acid side of the isoelectric point the cations of a salt have no effect on these properties of proteins, and on the alkaline side the anions have no effect. This all agrees with the results of the experiments mentioned in Chap. II.

Further, the experiments show that all the salts with anions of the same valency act quantitatively alike on the three properties of proteins on the acid side of the isoelectric point, and that all salts with cations of the same valency act alike on the alkaline side of the isoelectric point, but that the chemical nature of the ion is of no significance (except indirectly by influencing one of the crystalloidal properties of proteins, such as solubility or cohesion).

It seems that for any theory of adsorption the chemical nature of the ion of a salt should be of paramount importance. Those who adhere to the adsorption hypothesis have felt this, as is shown by their reluctance to abandon their belief in the reality of the Hofmeister ion series for swelling, osmotic pressure, and viscosity of proteins. Since this belief is no longer tenable, the conclusion is inevitable that the only ions which could possibly be adsorbed by a protein are the H and the OH ions. As a matter of fact, some believers in the adsorption hypothesis now seek refuge in this assumption. In the case of gelatin chloride they assume that the H ion is adsorbed by the gelatin; and in the case of Na gelatinate they assume that the OH ion is adsorbed by the gelatin. The titration and the combination curves in Chap. IV show that acids and alkalis combine stoichiometrically with proteins and the believers in the adsorption hypothesis would have to abandon Freundlich's adsorption formula for a new theory of a stoichiometrical adsorption of ions. Does it not appear simpler to assume that proteins are amphoteric electrolytes which combine stoichiometrically with acids and alkalis, just as do the amino-acids from which the proteins are built up? There is no doubt that the adsorption hypothesis is due to the unfortunate

historical accident that the colloidal behavior of the proteins was investigated before the methods of measuring the hydrogen ion concentration had been invented. If the titration and combination experiments in Chap. IV, the experiments on solubility in Chap. VI, and the experiments on the valency rule in Chap. VII and in this chapter had been known from the beginning, nobody would have thought of suggesting that proteins combine with acids and alkalies by adsorption. The continuation of the use of the term "adsorption" where we are plainly dealing with combination is only an indication of the well-known fact that habits of thought and habits of expression possess inertia. The futility of speaking of an adsorption of H ions by proteins becomes obvious when we try to visualize what goes on in this case in terms of the electron theory, as suggested by the ideas of Lewis, Langmuir, and Kossel. In the molecule of NH_3 the three hydrogen atoms have each one electron in common with the nitrogen atom. The nucleus of the nitrogen atom has, however, a sufficient electrostatic force to attract a fourth hydrogen ion, so that when HCl is added to NH_3 the H ion of the acid is attracted and held by the N atom, which is now surrounded by four

H
hydrogen ions HNH , while the Cl ions remain unaltered.
H

The proteins being built up from amino-acids have amino groups of the form HNH , in which the positive nucleus of the

R
nitrogen atom has also enough force left to attract a further H ion, so that when HCl is added the H ion of the acid is held electrostatically by the nucleus of the N atom. This leads to

H
the formation of the compound HNH .
R

In the light of such a visualization it would be difficult to state what the actual difference might be between the adsorption of the hydrogen ion of an acid by the amino group and the chemical combination of the hydrogen ion of the acid with the amino group.

9. APPENDIX

The belief in the validity of the Hofmeister series has given rise to a flood of speculations concerning the nature of physiological

and pathological processes. These speculations were, unfortunately, rarely supported by adequate experiments and when experiments were made the hydrogen ion concentrations were ignored, so that the basis of these speculations is always uncertain, if not positively wrong. Thus it has been suggested that muscular contraction is due to swelling caused by acid formation. This may or may not turn out to be correct, but the production of acid in the muscle can only lead to increased swelling if the pH inside the muscle is either at the isoelectric point or slightly (but not too far) below that of the isoelectric point of the protein responsible for the alleged swelling, since only in that case can the production of acid in the muscle increase the swelling. If the pH of the proteins of the resting muscle should be too far on the alkaline side of their isoelectric points, the production of acid in the muscle must diminish the swelling already existing in the resting muscle. It is obvious that we must know the isoelectric points of the proteins in the muscle, as well as the pH in the resting and the active muscle, before a discussion of the hypothesis becomes profitable.

It has been stated that digestion of a protein by pepsin is preceded by the swelling of the protein and that the effects of acid on swelling and pepsin digestion run parallel. This statement is wrong and is the consequence of the failure to measure the pH of the protein solutions. Northrop¹ has shown that the rate of digestion of gelatin by pepsin is the same at the same pH in the presence of HCl and of H₂SO₄, while the swelling is at pH 3.0 about twice as high in HCl as in H₂SO₄.

These errors of colloid chemists, due to the failure of measuring the hydrogen ion concentration would be bad enough, but they have been made worse by the addition of another error, namely, the failure to recognize the rôle which the cell membrane plays in life phenomena. A number of authors do not believe in the existence of such a membrane and hence have ascribed phenomena which are due to the selective permeability of membranes to an imaginary influence of acid on swelling. Thus it has been suggested that the adsorption of water by the striped muscle (and by other cells) in hypotonic solutions is due to a colloidal swelling caused by a hypothetical acid formation inside the cells,

¹ NORTHROP, J. H., *J. Gen. Physiol.*, vol. 5, p. 263, 1922-23.

but it can be shown that, if the solution is rendered isotonic by the addition of a sugar, the living muscle no longer absorbs water.¹ This proves that the absorption of water by living muscles (and other living cells) in hypotonic solution is due to the fact that these tissues or cells are surrounded by semi-permeable membranes and that the absorption of water by living striped muscles or cells in hypotonic solutions has no connection with colloidal swelling, since swelling is not depressed by sugar.

It has been stated that edema is due to the swelling of proteins inside the cells caused by acid formation. Not only have none of the measurements of the hydrogen ion concentrations required for such a hypothesis been made, but all critical experiments and all critical clinical observations show that edema is due to an increase of liquid in the spaces between tissues or cells, while there is no indication that edema is connected with colloidal swelling inside the cells.²

The enumeration of errors due to the fact that some colloidal authors prefer speculations to exact measurements, especially where the measurements require the use of the hydrogen electrode, might be continued, but it is hoped that these remarks may suffice.

¹ HÖBER, R., "Physikalische Chemie der Zelle und der Gewebe," 4th ed., p. 386, Leipsic and Berlin, 1914. LOEB, J., *Science*, vol. 37, p. 427, 1913.

² See HIRSCHFELDER, A. D., *Trans. Section Pharmacol. and Therapeutics, Am. Med. Assoc.*, p. 182, 1917. MOORE, A. R., *Am. J. Physiol.*, vol. 37, p. 220, 1915.

CHAPTER IX

THE INADEQUACY OF THE PRESENT THEORIES OF COLLOIDAL BEHAVIOR

When the era of colloidal speculation was ushered in it was hoped that it was the beginning of a new chemistry with laws different from the stoichiometric laws of crystalloids. We have seen that this difference does not exist for proteins, since proteins combine stoichiometrically with acids and alkalies, according to the laws of classical chemistry.

It was found further that genuine proteins, such as crystalline egg albumin or gelatin, are not held in solution by electrical double layers (believed to be due to preferential adsorption of ions), but apparently by the same forces which determine the solution of crystalloids. Both results seem natural in view of the fact that proteins are amino-acids in peptide linkage. It is possible that the proteins may also act like crystalloids in regard to other properties, such as surface tension, cohesion, and that type of viscosity which is common to all solutions.

If, then, proteins behave like crystalloids in regard to their chemical reactions, and often even in regard to their solubility, the question may be raised: In what direction does their behavior differ from that of crystalloids? To this the answer must be given that electrolytes affect certain properties of protein solutions and protein gels in an apparently specific way—at least as far as our present knowledge goes. The properties of proteins affected in this peculiar way are, as we have seen, the osmotic pressure and viscosity of protein solutions, and the swelling of protein gels. (Another property, namely, membrane potentials, will be added to these in the second part of this volume.)

It is a very striking fact that all of these three (or rather four) properties are affected in a similar way by electrolytes, which may be summarized as follows:

1. The addition of little acid (or alkali) to an isoelectric protein (crystalline egg albumin, gelatin, casein, edestin, serum globulin) increases the osmotic pressure, a certain type of viscosity (and also, as will be seen in the second part of this volume), the membrane potentials of protein solutions, and the swelling of protein gels, until a maximum is reached, after which the addition of further acid or alkali depresses these properties again.

2. This effect of acids and alkalies varies only with the valency of the anions of the acids and of the cations of the alkalies, but not with the chemical nature of these ions; ions of the same sign and valency, *e.g.*, Cl, NO₃, CH₃COO, H₂PO₄, HC₂O₄, etc., having the same influence on the above-mentioned properties of the protein, provided that the properties of the protein solutions are compared for the same pH and the same concentration of originally isoelectric protein, and provided that these ions have no secondary effects.

3. When the anion of the acid or the cation of the alkali is bivalent (*e.g.*, SO₄, Ca, Ba), the osmotic pressure, viscosity, and swelling of the protein are considerably less than when the ion is monovalent (*e.g.*, Cl, Br, NO₃, H₂PO₄, HC₂O₄, Na, K, etc.).

4. The addition of a neutral salt to a protein solution (which is not at the isoelectric point) depresses the osmotic pressure, viscosity (and P.D.) of the solutions and the degree of swelling of gels, and this effect increases with the valency of that ion of the salt which has the opposite sign of charge to that of the protein ion, but is independent of the chemical nature of the ion.

The colloidal literature has no quantitative explanation to offer for these effects. Zsigmondy makes an attempt to explain qualitatively the depressing influence of neutral salts on the osmotic pressure of a gelatin solution, by assuming that the addition of salt increases the degree of aggregation and hence diminishes the number of the particles in solution, the diminution in the number of particles leading to the lowering of osmotic pressure.¹ It is undoubtedly true that salts precipitate proteins and that precipitation is due to an increase in aggregation, but the salting out of gelatin from its aqueous solution is not determined by the ion with the opposite sign of charge to that of the protein ion, while we have seen that the depressing effect of a salt on the

¹ ZSIGMONDY, R., "Kolloidchemie," 2d ed., p. 342, Leipsic, 1918.

osmotic pressure of gelatin solutions is determined by the ion with the opposite sign of charge to that of the protein ion. In other words, the salting out of gelatin from its aqueous solution is a process of an entirely different character from the lowering of the osmotic pressure of a protein solution by a neutral salt. It is, therefore, impossible to explain the latter process by the former.

Moreover, the attempt to explain the depressing effect of the addition of salts on the basis of the micella theory fails completely in the case of the other properties of protein solutions, which are equally depressed by them as the osmotic pressure, namely, the viscosity and the P.D. and the swelling of protein gels.

We shall see in Chap. XVI that, if the state of aggregation increases in a gelatin solution—*i.e.*, if isolated protein molecules or ions unite to form a larger aggregate—the viscosity of the solution is thereby increased, for the reason that these aggregates occlude comparatively large quantities of water whereby the relative volume occupied by the gelatin in the solution is increased. This increase in the volume of the micellæ at the expense of water leads, as will be seen, to an increase in viscosity. Hence if we assume that the addition of a salt increases the degree of aggregation in protein solution, it would follow that this should result in an increase of viscosity, while the addition of salt in reality depresses the viscosity. The attempt to explain the depressing influence of salts on the osmotic pressure and viscosity of protein solutions on the basis of the aggregation theory leads, therefore, to conclusions which are in contradiction with the actual facts.

Pauli makes an attempt to explain the fact that little acid or alkali increases and that more acid or alkali diminishes the viscosity of albumin solutions by assuming that the acid leads to formation of a protein salt (which is correct) and that the ionization leads to increased hydration of the protein ion. The depressing effect of the addition of more acid he explains by the assumption that the degree of ionization of the protein salt is depressed by the addition of more acid or by the addition of salt. These speculations have at least the advantage over the speculations on dispersion that they can be put to a quantitative test. When this is

done, it is found that they are not tenable for the viscosity of gelatin solutions.

In order to explain how the ionization of proteins could increase the viscosity of protein solutions Pauli assumed that the protein ion is surrounded by an enormous shell of water, which he thinks is lacking in the case of the non-ionized protein molecule.¹ The shell of water might prevent the coalescence of the protein ions and hence might cause a higher degree of dispersion. On the basis of the hydration theory, we can find a qualitative explanation of the peculiar pH curves in the following way: At the isoelectric point protein is in a non-ionized condition and no hydration occurs. Hence the degree of dispersion of particles and the osmotic pressure are a minimum at this point and the viscosity and swelling should also be a minimum, since swelling might be directly due to the existence of this water jacket, and viscosity should also increase with the mass of water surrounding each particle. If an acid, *e.g.*, HCl, is added to isoelectric gelatin, the latter will be transformed into gelatin chloride which, being a salt, is strongly dissociated. The more acid is added the more gelatin is transformed into gelatin chloride. We have shown in Chap. VII that the curves for osmotic pressure, swelling, and viscosity reach a maximum at a pH varying between 3.5 and 2.8, and that they then drop. On the basis of observations on blood albumin Pauli assumes that the drop is due to a repression of the degree of electrolytic dissociation of the gelatin chloride (or any protein-acid salt) through the addition of more acid on account of the common anion. It should, however, be mentioned that Pauli² and Manabe and Matula³ state that the maximum of the curves occurs not at pH between 3.5 and 2.8, but at pH 2.1 or 2.0.

Hitchcock determined the pH at which maximal ionization occurs for a number of different protein chlorides.⁴ These maxima are given in the last row of Table X. In the upper rows are given the pH where the maximal osmotic pressure, swelling, viscosity,

¹ PAULI, W., *Fortschritte naturwiss. Forschung*, vol. 4, p. 223, 1912; "Kolloidchemie der Eiweisskörper," Dresden and Leipsic, 1920.

² PAULI, W., "Kolloidchemie der Eiweisskörper," Dresden and Leipsic, 1920.

³ MANABE, K., and MATULA, J., *Biochem. Z.*, vol. 53, p. 369, 1913.

⁴ HITCHCOCK, D. I., *J. Gen. Physiol.*, vol. 5, p. 383, 1922-23.

and membrane potentials for the same proteins occur. The maximum of ionization occurs at a much lower pH than that for the maximum of other properties of the same protein. It is not possible to assume with Pauli that the depressing effect of higher concentrations of HCl on viscosity, osmotic pressure, or swelling is due to a repression of ionization of gelatin chloride.

TABLE X.—pH VALUES CORRESPONDING WITH MAXIMA IN VARIOUS PROPERTIES OF 1 PER CENT PROTEIN CHLORIDE SOLUTIONS

	Gelatin	Egg albumin	Casein	Edestin	Serum globulin
Osmotic pressure.....	3.4	3.4	3.0	3.0	3.0
Swelling.....	3.2				
Viscosity.....	2.9	No maximum	3.0		
Membrane potential..	4.1	3.6	3.6	4.3	3.4
Ionization.....	1.4	1.6	2.2	1.6	2.2

The hydration hypothesis can be put to a direct test by determining the specific conductivity of solutions of protein salts, *e.g.*, gelatin chloride, albumin chloride, etc. Since according to the hydration hypothesis only the protein ion undergoes hydration, the variation in the osmotic pressure, swelling, and viscosity should be accompanied by a corresponding variation in the concentration of protein ions in solution. If, therefore, the specific conductivity of gelatin chloride is measured at varying pH but equal concentrations of originally isoelectric gelatin, the curves representing the values found for conductivity of the protein should run parallel with the curves for the osmotic pressure, swelling, and viscosity; moreover, the curve for the conductivity of gelatin sulphate should be only about half as high as the curve for the specific conductivity of gelatin chloride, while the curve for the specific conductivity of gelatin oxalate should be almost but not quite as high as that for gelatin chloride. The experiments show that this is not the case.

The concentration of ionized gelatin in solution can be determined with the aid of conductivity measurements of the solution of a gelatin salt, *e.g.*, gelatin chloride, by deducting the conduc-

tivity of the free HCl in the solution from the total conductivity of the gelatin solution, since the gelatin chloride solution prepared by the writer's method from washed powdered isoelectric gelatin contains practically no other electrolyte except the free HCl and the gelatin chloride. This was proved by ash determinations and by the fact that a solution of isoelectric gelatin prepared

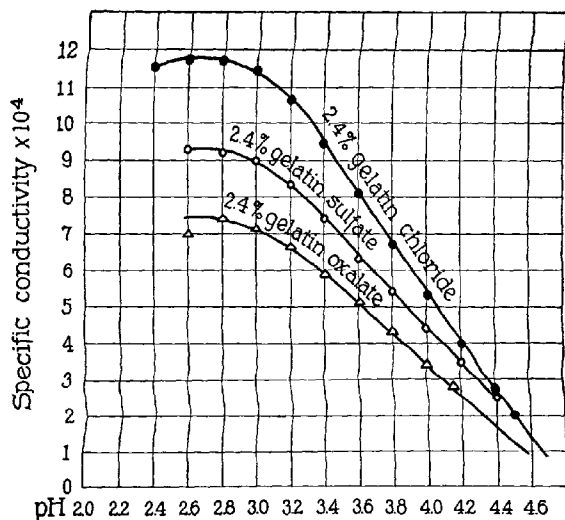


FIG. 55.—Curves for the specific conductivity of 2.4 per cent solutions of gelatin chloride, sulphate, and oxalate, showing the entirely different character of these curves from that of the osmotic pressure curves in Figs. 26 and 27.

according to our method of washing has practically a conductivity of zero. The method of procedure was as follows:

Solutions of different gelatin-acid salts were prepared in two different concentrations of originally isoelectric gelatin, 0.8 and 2.4 per cent. The specific conductivities of these gelatin-acid salts were determined at different pH. The conductivities of pure aqueous solutions of the same acids at different pH were also measured. In both cases the conductivities were plotted as ordinates over the pH as abscissæ. By deducting the values for the specific conductivity of the pure aqueous solution of an acid

from the values for the total specific conductivity of the gelatin-acid solution of the same pH, the curve for the specific conductivity of the gelatin-acid salt for that pH is obtained.¹

Figure 55 shows that the curves representing the percentage of ionized gelatin in gelatin chloride resemble the combination curves in Fig. 8, since in both cases there is a gradual rise in the concentration of ionizable protein at a pH below that of the isoelectric point, but no maximum followed by a drop at pH 3.4 or 3.0. In fact, no drop in the conductivity curves occurs until

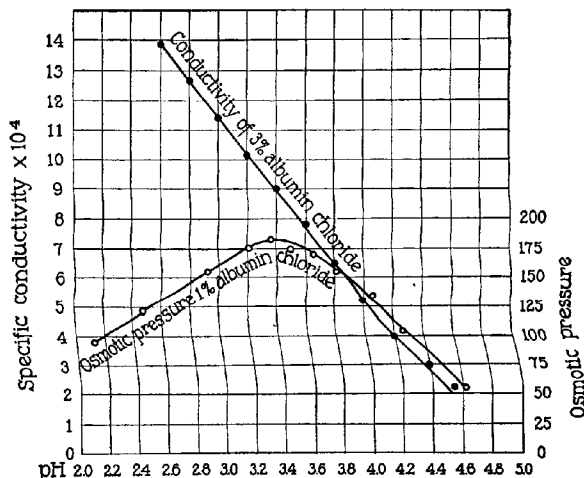


FIG. 56.—Comparison of conductivity curve and osmotic pressure curve for albumin chloride, showing the entirely different character of the two curves.

the pH falls below 2.0. This was found not only by the writer but also by Northrop. It is, therefore, impossible to attribute the depression in the osmotic pressure curves which commences when the pH falls below 3.4 to a repression of the ionization of the protein salt.

This is still more clearly illustrated by Fig. 56. The lower curve gives the osmotic pressure of a 1 per cent solution of albumin chloride, showing a maximum at pH 3.4. The other curve is the conductivity curve for a 3 per cent solution of albumin chlo-

¹ LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 247, 1920-21.

ride also plotted over the pH of the solution as abscissæ. It is quite obvious that the conductivity curve has no maximum at pH 3.4 or even pH 3.0 but that it continues to rise to a pH of 2.6. No drop occurs until the pH is 2.0 or less. It is, therefore, obvious that the characteristic drop in the curves for osmotic pressure, viscosity, or swelling, which occurs when the pH is 3.4 or 3.0, cannot be explained on the assumption that in an acid of a pH below 3.4 or 3.0 a steep drop of the specific conductivity occurs.

Neither is it possible to explain the valency effect on the basis of Pauli's theory. We have seen that the curve for the osmotic pressure, viscosity, or swelling of gelatin chloride is at the maximum about twice as high as that for gelatin sulphate, and the curve for gelatin oxalate is but slightly lower than that for gelatin chloride. If this phenomenon is to be explained on the basis of Pauli's theory, it should be necessary to prove that the conductivity of gelatin chloride is about the same as that for gelatin oxalate but considerably higher than that for gelatin sulphate. Figure 55 gives measurements of the conductivities of these three gelatin salts, showing that the conductivity of gelatin sulphate is but slightly less than that of gelatin chloride and a little higher than that of gelatin oxalate.

Pauli's hydration theory rests on an assumption made by Kohlrausch that the difference in the mobility of ions is due to molecules of water being dragged along with the migrating ion. Lorenz,¹ Born,² and others have come to the conclusion that while Kohlrausch's idea is probably correct for monatomic ions it cannot be correct for large polyatomic ions. This would exclude the assumption of the high degree of hydration of the protein ions on which Pauli's theory rests. It is, therefore, quite obvious that Pauli's theory cannot account for the depressing influence of electrolytes on the physical properties of proteins.

A mathematical and a quantitative explanation of the influence of electrolytes on the colloidal behavior of protein solutions can be given on the basis of Donnan's theory of membrane equilibria. The proof for this statement will be given in the second part of this book.

¹ LORENZ, R., *Z. Elektrochem.*, vol. 26, p. 424, 1920; "Raumerfüllung und Ionenbeweglichkeit," Leipzig, 1922.

² BORN, M., *Z. Elektrochem.*, vol. 26, p. 401, 1920.

PART II
THEORY OF THE COLLOIDAL BEHAVIOR
OF PROTEINS

CHAPTER X

INTRODUCTORY REMARKS ABOUT THE THEORY

A theory of the colloidal behavior of proteins is not concerned with the purely crystalloidal properties of proteins, such as chemical combination and solubility. These two properties were dealt with in the first part of this work. Neither are we concerned here with other properties of protein solutions, such as surface tension, which may also be purely crystalloidal in character. We are concerned with two problems, namely, first, why have electrolytes a similar effect on three entirely different properties of proteins, osmotic pressure, swelling, and a certain type of viscosity (as shown in Chaps. VII and VIII); and second, why does this effect show the characteristics enumerated already in the last chapter, which we will repeat here for the convenience of the reader?

1. The addition of little acid (or alkali) to an isoelectric protein (gelatin, crystalline egg albumin, casein, edestin, serum globulin) increases the osmotic pressure, a certain type of viscosity of protein solutions, and the swelling of protein gels, until a maximum is reached, after which the addition of further acid or alkali depresses these properties again (Fig. 57).

2. This effect of acids and alkalies varies with the valency of the anion of acids and of cations of alkalies, but not with the chemical nature of these ions; ions of the same sign and valency, *e.g.*, Cl, NO₃, CH₃COO, H₂PO₄, HC₂O₄, etc., having the same influence on the osmotic pressure and the other properties, provided the properties of the protein solutions or gels are compared for the same pH and the same concentration of originally isoelectric protein (Fig. 57).

3. When the anion of the acid or the cation of the alkali is bivalent (*e.g.*, SO₄, Mg, Ca, Ba), the osmotic pressure, viscosity, and swelling of the protein are considerably less than when the ion is monovalent (*e.g.*, Cl, Br, NO₃, H₂PO₄, HC₂O₄, Li, Na, K, NH₄, etc.—see Fig. 57).

4. The addition of a neutral salt to a protein solution (not at the isoelectric point) depresses the osmotic pressure and viscosity of the protein solutions, and the degree of swelling of gels, and this effect increases with the valency of that ion of the salt which has the opposite sign of charge to that of the protein ion, but is independent of the chemical nature of the ion.

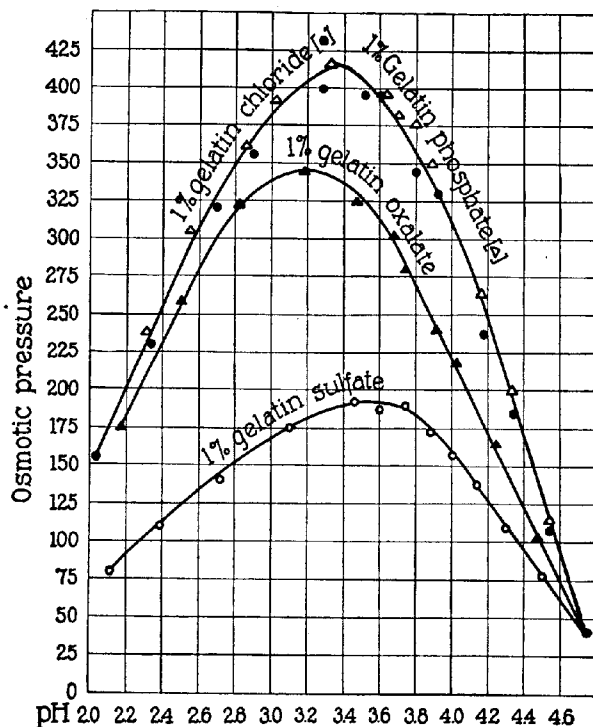


FIG. 57.—Observed variation of osmotic pressure with hydrogen ion concentration and valency of the anion of the acid.

The similarity of the influence of electrolytes on the three physical properties mentioned (osmotic pressure, viscosity, and swelling) suggests that we may be dealing with the same fundamental property in all three cases, and we shall see that this is

true and that the property in question is the osmotic pressure. We may, therefore, in a preliminary discussion confine our attention to this latter property. How then does it happen that, if we add some acid, *e.g.*, HCl, to a solution of isoelectric protein, the osmotic pressure increases at first with increasing concentration of the hydrogen ions of the protein solution until a maximum is reached (which lies in the case of gelatin or egg albumin at a pH of about 3.4), while with the addition of more acid the osmotic pressure diminishes again (Fig. 57)? Some¹ colloid chemists took it for granted that this is due to some influence of the electrolyte on some colloidal property of the protein, such as the degree of dispersion of its micellæ in solution. While such an effect of acids on the protein may exist, we shall see that there exists a different cause for the peculiar influence of the pH on the osmotic pressure of protein solutions. This becomes clear through measurements of the pH of the protein chloride solution and the outside aqueous solution with which the protein solution is in equilibrium. Let us assume that a collodion bag is filled with a gelatin chloride solution of pH 3.0 and that this collodion bag is submerged in aqueous solution of HCl, originally also of pH 3.0, but free from protein. When osmotic equilibrium is established between the protein solution and the outside aqueous solution, it is found that the pH is no longer the same inside and outside the protein solution. Hence, the osmotic pressure of the protein solution indicated by the manometer is not exclusively the osmotic pressure of the protein solution alone, but is partly determined by the difference in the concentration of crystalloidal ions (namely, H and Cl ions in the case of a solution of gelatin chloride) inside and outside the protein solution. This is true not only for protein solutions at pH 3.0, but at any pH (except that of the isoelectric point of the protein). Hence, before we can speculate on the possible cause of the influence of the pH (or of electrolytes in general) on the osmotic pressure of a protein solution, we must correct the observed osmotic pressure of the protein solution for this difference in the concentration of the crystalloidal ions inside and outside the protein solution. The question arises as to how to calculate the necessary correction. This is only possible if we possess the

¹ ZSIGMONDY, R., "Kolloidchemie," 2d ed., Leipsic, 1918.

mathematical theory for this inequality of distribution of crystalloidal ions inside and outside the protein solution, which the pH and pCl measurements show to exist. In order to arrive at this theory it was necessary to introduce measurements of a quantity which had thus far received little attention in the colloidal literature, namely, membrane potentials. The writer found that there exists a difference of potential between a solution of a protein salt, *e.g.*, gelatin chloride, and an aqueous solution free from protein at the time osmotic equilibrium is established between the protein solution and the outside aqueous solution. In our experiment the solution of protein salts is inside a collodion bag submerged in an aqueous solution free from protein. After the membrane potentials between the solution of the protein salt, *e.g.*, gelatin chloride, and the outside aqueous solution had been measured with indifferent and identical electrodes, the attempt was made to find out whether this P.D. was in any way connected with the difference in the hydrogen ion concentration between the protein solution and the outside aqueous solution at equilibrium. Measurements of the P.D. between the protein solution and the outside aqueous solution with the hydrogen electrode showed that this latter P.D. is identical with the membrane potentials measured by the indifferent electrodes.¹ Since this agreement was to be expected on the basis of Donnan's theory of membrane equilibria, which was discussed in the first chapter, but on no other theory, it follows that the difference in the concentration of diffusible electrolytes inside and outside the membrane can be calculated from Donnan's equation for membrane equilibria. This makes it possible, as we shall see, to derive the influence of electrolytes on the colloidal behavior of proteins quantitatively and mathematically.

¹ This was by no means to be taken for granted, as anyone familiar with Beutner's work on "Die Entstehung elektrischer Ströme in lebenden Geweben" will admit. Only two conditions can account for the equality of the membrane potentials and hydrogen electrode potentials in this case, namely, the fact that the proteins form ionizable salts with acids and alkalis, and that the protein ions cannot diffuse through the membrane which is permeable to the small crystalloidal ions. This seems to have been overlooked by A. V. Hill (*Proc. Roy. Soc.*, vol. 102, p. 705, 1923), as was pointed out by Hitchcock (*J. Gen. Physiol.*, vol. 5, p. 383, 1922-23).

CHAPTER XI

MEMBRANE POTENTIALS¹

METHODS OF MEASUREMENT AND THE INFLUENCE OF THE HYDROGEN ION CONCENTRATION ON MEMBRANE POTENTIALS

When a solution of a protein salt, *e.g.*, 1 per cent gelatin chloride, is separated from distilled water by a collodion membrane, a potential difference exists across the membrane between the gelatin chloride solution and the outside solution with which it is in equilibrium. If this P.D. is measured with the aid of a Compton quadrant electrometer with saturated KCl calomel electrodes, it is found that the P.D. is influenced in a similar way by electrolytes as the osmotic pressure, swelling, and viscosity (see Fig. 59 in this chapter). This in itself would only mean the addition of another property varying in the same characteristic way as osmotic pressure, or swelling, or viscosity of proteins under the influence of electrolytes, if it were not for the fact that we can correlate the variations of the new property with the Donnan equilibrium.

It is necessary to give a brief description of the method of measuring the P.D. across the membrane. Suppose that the protein in solution is gelatin chloride containing 1 gm. of originally isoelectric gelatin in a 100-c.c. solution. Such solutions of gelatin chloride are put into collodion bags closed with rubber stoppers which are perforated with glass tubes serving as manometers, as described in the osmotic pressure experiments of Chap. VII. These collodion bags filled with the gelatin chloride solution are submerged in beakers containing 350 c.c. of aqueous HCl solution of originally the same pH as that of the gelatin chloride solution, but free from gelatin. The experiments last 20 hours or more at 24°C. to allow the establish-

¹ This chapter is based on LOEB, J., *J. Gen. Physiol.*, vol. 3, pp. 557, 667, 1920-21; vol. 4, pp. 351, 463, 617, 741, 1921-22; *J. Am. Chem. Soc.*, vol. 44, p. 1930, 1922.

ment of osmotic equilibrium between the two solutions (which requires only about 6 hours under the conditions of the experiments). After 20 hours or more the P.D. between the gelatin solution (which we call the inside solution) and the aqueous solution (which we call the outside solution) is measured across the membrane with the aid of a Compton electrometer, giving a deviation of about 2 mm. on the scale for 1 millivolt at a distance of about 2 m. The two electrodes leading to the electrometer are identical (Fig. 58). They are

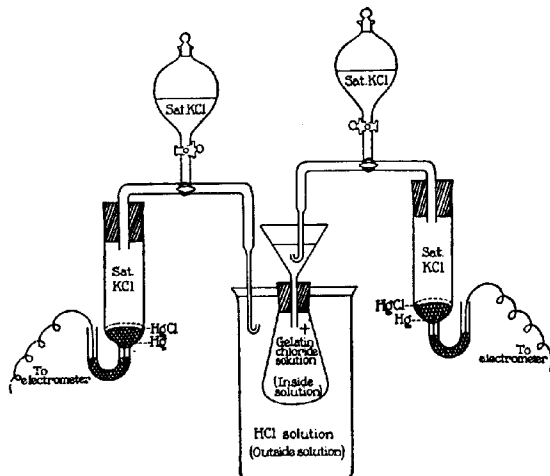


FIG. 58.—Method of measuring the P.D. between gelatin chloride solution in a collodion bag and the outside HCl solution in beaker.

calomel electrodes filled with saturated KCl solution. One electrode dips through a capillary glass tube into the gelatin solution, the other also through a capillary glass tube into the outside solution. In order to allow the electrode to dip into the gelatin solution, the glass tube serving as a manometer is replaced by a funnel, as shown in the figure. In the figure the upper level of the gelatin solution is in the funnel. This is not really necessary, but it is convenient and is accomplished by allowing the collodion bag to press against the glass wall of the beaker con-

taining the outside solution. As a minor but convenient accessory, each electrode is connected with a reservoir of saturated KCl solution, which makes it possible to let the KCl solution in the capillary flow out after each measurement, so that the electrode is always clean for each new measurement.¹ What was measured in this way was, therefore, the electromotive force of the following cell:

calomel electrode	saturated KCl	outside solution HCl —	collodion mem- brane	inside solution gelatin chloride +	saturated KCl	calomel electrode
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It is found that in this cell the gelatin chloride solution has a positive charge and the outside solution a negative charge and that the membrane potential varies with the pH of the gelatin chloride solution, as indicated in Fig. 59. It is also found that the P.D. of gelatin phosphate solutions is practically identical with the P.D. of gelatin chloride solutions of the same pH and that both are considerably higher (about 50 per cent higher, as we shall see) than the P.D. of gelatin sulphate solutions. We shall also see that the addition of a neutral salt to the gelatin chloride solution depresses the P.D., and the more so the higher the valency of the anion of the salt. In other words, electrolytes influence the membrane potential between gelatin chloride solution and outside solution in a way similar to that in which they influence the osmotic pressure and the viscosity of the same solution. It becomes, therefore, of considerable importance to find out the origin of this membrane potential. We intend to show that the P.D. is due to the establishment of a Donnan equilibrium between the gelatin chloride solution and the outside aqueous solution (free from gelatin).

In our experiment a collodion bag filled with a 1 per cent solution of gelatin chloride is submerged in a beaker containing a solution of HCl (without gelatin) of originally the same pH

¹ In some experiments the measurements were made through the manometer tube without releasing the hydrostatic pressure. This P.D. was the same as with the method described.

as that of the gelatin solution. In this case we have free HCl inside as well as outside, but in addition we have inside the collodion bag a gelatin chloride solution which ionizes into Cl and a positive gelatin ion. The gelatin ion is unable to diffuse through the collodion membrane, but the H and Cl ions can

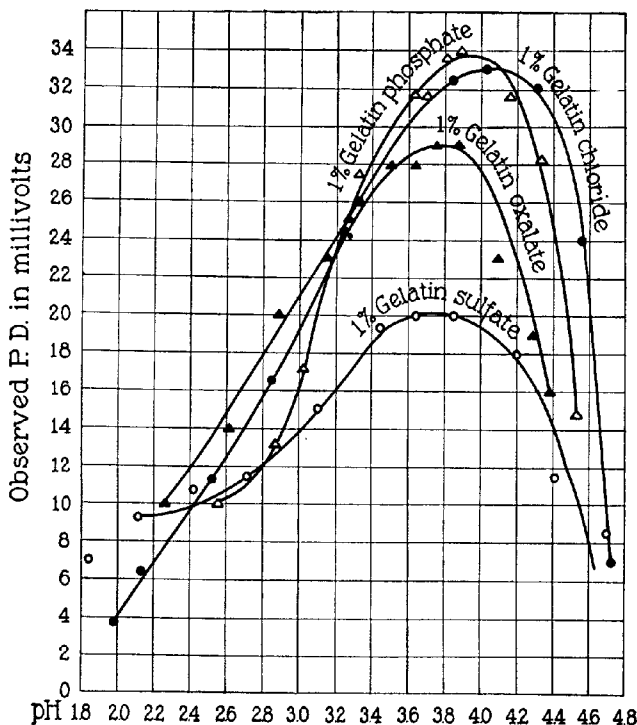


FIG. 59.—Influence of pH and valency of anion on P.D. of solutions of different gelatin-acid salts. The curves in Fig. 59 are similar to (but not identical with) those in Fig. 57.

diffuse freely through the membrane. On the basis of Donnan's theory of membrane equilibria, in this case an equilibrium condition should be established in which the product of the concentrations of the H and Cl ions in the outside solution equals the product of the concentrations of the H and Cl ions inside. This

equilibrium is expressed by the following equation, which was first used by Procter and Wilson for the distribution of free HCl between a jelly of solid gelatin chloride and surrounding water, but which holds also for the case where the gelatin chloride is in solution and separated from the outside solution by a collodion membrane impermeable to gelatin ions, but permeable to H and Cl ions,

$$x^2 = y(y + z) \quad (1)$$

where x is the molar concentration of H and Cl ions in the outside solution, y the molar concentration of the H and Cl ions of the free acid inside the gelatin solution, and z the molar concentration of the Cl ions in combination with the gelatin. (For the sake of simplification, complete electrolytic dissociation of HCl and gelatin chloride is assumed.)

It is our task to show that these membrane potentials across the membrane observed with indifferent calomel electrodes are determined by the Donnan equilibrium. This proof can be furnished in the following way.

Donnan has shown that, as a consequence of his formula, the membrane potential observed must be

$$\text{P.D.} = \frac{RT}{F} \log \frac{x}{y} = \frac{RT}{F} \log \frac{y + z}{x}.$$

In other words, the membrane potential should be determined by the difference in the concentration of any diffusible ion, *e.g.*, the hydrogen ions inside and outside at equilibrium. This difference of potential due to the difference in the pH outside and inside at equilibrium can be measured directly with the hydrogen electrode, and this P.D. measured with the hydrogen electrode (which we will call the hydrogen electrode P.D.) should be equal to the membrane potential across the membrane measured with indifferent electrodes. In the case of the hydrogen electrode potential the electromotive force of the following cell is measured.

hydrogen electrode	outside solution HCl	saturated KCl	inside protein solution	hydrogen electrode
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In this measurement the sign of the P.D. is the reverse of that of the membrane potential.

Instead of measuring the hydrogen electrode potential between the protein solution and the outside aqueous solution, as indicated by the diagram, the hydrogen electrode potentials of the two solutions were measured successively against the standard calomel cell, for the reason that it was necessary to plot the values of both the membrane potential as well as the hydrogen electrode potential over the pH of the protein solution and this quantity had, therefore, to be measured separately.

The pH of the inside and outside solutions was measured after the establishment of equilibrium. Since pH inside is $-\log y$ and pH outside $-\log x$, and since the experiments were made at 24°C., the values for the hydrogen electrode potentials are given as 59 (pH inside minus pH outside) millivolts. These values for the hydrogen electrode potentials agree, within the limits of accuracy, with the values for the membrane potentials measured with the indifferent calomel electrodes, and this leaves no doubt that the membrane potentials are caused by the difference in the concentration of hydrogen ions on the opposite sides of the membrane, as Donnan's theory demands.

Tables XI, XII, and XIII show the existence of this agreement between membrane potentials and hydrogen electrode potentials at different pH of the gelatin solution. The upper two rows give the pH inside and outside at equilibrium as measured with the hydrogen electrode. The third horizontal row gives the difference pH inside minus pH outside, and the fourth row gives the hydrogen electrode potentials, *i.e.*, 59 (pH inside minus pH outside). The last row gives the observed membrane potentials as plotted in Fig. 59.

If the reader will compare the membrane P.D. and the hydrogen electrode P.D. in the last two rows of Tables XI, XII, and XIII, he will notice that the difference is practically never more than 2 millivolts, and generally less. This agreement is within the limits of accuracy of measurements and leaves no doubt that the Donnan equilibrium is responsible for the influence of acids on the membrane potentials of protein solutions.

It can be shown, furthermore, that Donnan's theory permits us to visualize the influence of pH on the membrane potentials of protein solutions.

TABLE XI.—INFLUENCE OF THE HYDROGEN ION CONCENTRATION ON HYDROGEN ELECTRODE POTENTIALS AND ON MEMBRANE POTENTIALS OF GELATIN CHLORIDE SOLUTIONS AT EQUILIBRIUM

	Cubic centimeters 0.1 N HCl contained in 100 c.c. of 1 per cent isoelectric gelatin										
	1	2	4	6	8	10	12.5	15	20	30	50
pH inside.....	4.56	4.31	4.03	3.85	3.53	3.25	2.85	2.52	2.13	1.99	1.79
pH outside.....	4.14	3.78	3.44	3.26	2.87	2.81	2.53	2.28	2.00	1.89	1.72
pH inside minus pH outside.....	0.42	0.53	0.59	0.59	0.46	0.44	0.32	0.24	0.13	0.10	0.07
Hydrogen electrode P.D. (millivolts).....	+24.7	+31.0	+34.5	+34.5	+27.0	+25.8	+18.8	+14.0	+7.6	+5.9	+4.1
Membrane P.D. (millivolts).....	+24.0	+32.0	+33.0	+32.5	+26.0	+24.5	+16.5	+11.2	+6.4	+4.8	+3.7
											+2.3
											+3.7
											+2.1

TABLE XII.—INFLUENCE OF THE HYDROGEN ION CONCENTRATION ON HYDROGEN ELECTRODE POTENTIALS AND ON MEMBRANE POTENTIALS OF GELATIN PHOSPHATE SOLUTIONS AT EQUILIBRIUM

	Cubic centimeters $m/10$ H_2PO_4 contained in 100 c.c. of 1 per cent isoelectric gelatin													
	0	1	2	4	6	7	8	10	12.5	15	20	30	40	50
pH inside.....	4.70	4.54	4.31	3.98	3.68	3.56	3.38	3.24	3.02	2.67	2.42	2.12	1.92	1.74
pH outside.....	4.70	4.10	3.77	3.40	3.14	3.04	2.90	2.80	2.66	2.39	2.22	1.98	1.83	1.67
pH inside minus pH outside.....	0.09	0.44	0.54	0.58	0.54	0.52	0.48	0.44	0.36	0.28	0.20	0.14	0.09	0.07
Hydrogen electrode P.D. (millivolts).....	+5.3	+25.8	+31.7	+34.0	+31.7	+30.5	+28.0	+25.8	+21.2	+16.4	+11.7	+8.2	+5.3	+4.1
Membrane P.D. (milli- volts).....	+5.7	+27.0	+29.0	+30.0	+30.6	+29.6	+26.5	+24.4	+22.3	+17.7	+15.6	+11.4	+9.9	+7.3

TABLE XIII.—INFLUENCE OF THE HYDROGEN ION CONCENTRATION ON HYDROGEN ELECTRODE POTENTIALS AND ON MEMBRANE POTENTIALS OF GELATIN SULPHATE SOLUTIONS AT EQUILIBRIUM

Cubic centimeters 0.1 N H ₂ SO ₄ contained in 100 c.c. of 1 per cent isoelectric gelatin														
	0	1	2	4	6	7	8	10	12.5	15	20	30	40	50
pH inside.....	4.76	4.52	4.34	3.98	3.73	3.49	3.41	3.12	2.78	2.47	2.16	2.06	1.84	1.57
pH outside.....	4.61	4.20	3.99	3.60	3.38	3.18	3.14	2.88	2.61	2.35	2.09	2.00	1.80	1.54
pH inside minus pH outside..	0.15	0.32	0.35	0.38	0.35	0.31	0.27	0.24	0.17	0.12	0.07	0.06	0.04	0.03
Hydrogen electrode P.D. (millivolts).....	+8.8	+18.8	+20.5	+22.2	+20.5	+18.1	+15.8	+14.0	+10.0	+7.0	+4.1	+3.5	+2.4	+1.8
Membrane P.D. (millivolts)...	+6.3	+16.3	+18.4	+19.0	+19.0	+17.4	+15.8	+13.7	+10.5	+8.4	+7.4	+5.8	+4.7	+3.7

It follows from the equilibrium equation,

$$x^2 = y(y + z), \quad (1)$$

that

$$x = \sqrt{y(y + z)}.$$

Substituting $\sqrt{y(y + z)}$ for x in the term $\frac{x}{y}$, we get

$$\frac{\sqrt{y(y + z)}}{y} = \sqrt{\frac{y + z}{y}} = \sqrt{1 + \frac{z}{y}}.$$

Hence at 18°C. the P.D. should be $= \frac{58}{2} \log \left(1 + \frac{z}{y}\right)$ millivolts.

We will now show that from the term $\sqrt{1 + \frac{z}{y}}$ the influence of pH on the P.D. as expressed in the curves of Fig. 59 can be derived.

When we add little HCl to isoelectric gelatin we increase the amount of gelatin chloride formed, and hence the value of z . This follows from the combination curves between gelatin and acid discussed in Chap. IV. Hence, the P.D. should increase when little acid is added to isoelectric gelatin, since the P.D. depends on $\log \left(1 + \frac{z}{y}\right)$. The addition of acid also increases the value of y , but z and y will not increase at the same rate. When little acid is added to isoelectric gelatin, the value of z rises more rapidly than the value of y , since at first the greater part of the acid enters in combination with the gelatin, while when more acid is added the reverse happens, since when a considerable part of the gelatin is already transformed into salt, only a small fraction of the acid added will be used further to increase the formation of gelatin salt. This is obvious from Table XIV comparing the variations of z and y upon the addition of increasing quantities of HCl to isoelectric gelatin.

The fifth vertical column of the table shows that at first the value $\frac{z}{y}$ increases with increasing addition of acid until pH = 4.03,

and that with the addition of more acid the value $\frac{z}{y}$ diminishes again. A comparison of the last and second last vertical columns shows that the observed and calculated P.D. agree.

TABLE XIV

Cubic centimeters 0.1 N acid in 100 c.c. of 1 per cent originally isoelectric gelatin	pH of gelatin solution at equilibrium	Conc. $y \times 10^4$ N	Conc. $z \times 10^4$ N	$\frac{z}{y}$	$\log \left(1 + \frac{z}{y} \right)$	P.D. calculated from $29 \log \left(1 + \frac{z}{y} \right)$ millivolts	P.D. observed, millivolts
1.0	4.56	2.7	16.5	6.1	0.8513	24.7	24.0
2.0	4.31	4.9	51.4	10.5	1.0607	30.7	32.0
4.0	4.03	9.3	132.5	14.3	1.1847	34.4	33.0
6.0	3.85	14.1	200.0	14.2	1.1818	34.3	32.5
8.0	3.33	46.8	343.0	7.3	0.9191	26.1	26.0
10.0	3.25	56.2	372.0	6.6	0.8808	25.5	24.5
12.5	2.85	141.0	477.0	3.4	0.6435	18.7	16.5
15.0	2.52	302.0	608.0	2.0	0.4771	13.8	11.2
20.0	2.13	741.0	609.0	0.82	0.2601	7.5	6.4

Donnan's equilibrium equation thus explains why the P.D. rises at first when HCl is added to isoelectric gelatin until the pH is 4.03, and why the P.D. drops when the pH falls below 3.8.

THE VALENCY EFFECT

Figure 59 shows that the curves of membrane potentials for gelatin chloride and phosphate are considerably higher than the curve for gelatin sulphate. The same valency effect was observed for the osmotic pressure curves, the viscosity curves, and the curves for swelling. It can be shown that the Donnan theory demands that for the same pH and the same concentration of originally isoelectric gelatin the membrane potentials of gelatin chloride and gelatin sulphate should stand in the ratio of 3:2, and it is one of the most convincing proofs of the correctness of the theory that the observations agree with this postulate.

The proof that the membrane potentials of the solutions of the two gelatin salts must show the ratio of 3:2 is as follows:

The equilibrium equation for gelatin chloride is of the second degree, namely,

$$\frac{x}{y} = \frac{y+z}{x};$$

and we have just seen that by proper substitution the

$$\text{P.D.} = \frac{58}{2} \log \left(1 + \frac{z}{y} \right) \text{ millivolts.}$$

The equilibrium equation which is of the second degree when the anion is monovalent becomes of the third degree when the anion is bivalent, *e.g.*, SO_4 , in the case of gelatin sulphate. Let x be the molar concentration of hydrogen ions in the outside solution, y the molar hydrogen ion concentration in the inside solution; then $\frac{x}{2}$ is the molar concentration of the SO_4 ions in the outside, and $\frac{y}{2}$ the molar concentration of the SO_4 ions of the free H_2SO_4 in the inside (gelatin) solution. The concentration of SO_4 ions in combination with gelatin becomes $\frac{z}{2}$. Since SO_4 dissociates into two hydrogen ions and one SO_4 ion, and since in the case of membrane equilibria the products of each pair of oppositely charged ions must be the same on opposite sides of the membrane, the equilibrium equation becomes

$$\begin{aligned} x^2 \left(\frac{x}{2} \right) &= y^2 \frac{(y+z)}{2} \\ x^3 &= y^2(y+z) \\ x &= \sqrt[3]{y^2(y+z)}. \end{aligned}$$

The value which interests us is $\frac{x}{y}$, *i.e.*, the ratio of the hydrogen ion concentrations.

Substituting $\sqrt[3]{y^2(y+z)}$ for x in $\frac{x}{y}$, we get

$$\frac{x}{y} = \sqrt[3]{\frac{y^2(y+z)}{y^3}} = \sqrt{\frac{y+z}{y}}.$$

The P.D. is, therefore, in the case of gelatin sulphate,

$$\text{P.D.} = \frac{58}{3} \log \left(1 + \frac{z}{y} \right) \text{ millivolts,}$$

while in the case of gelatin chloride it is

$$\text{P.D.} = \frac{58}{2} \log \left(1 + \frac{z}{y} \right) \text{ millivolts.}$$

Hence the membrane potentials of gelatin sulphate solutions should be only two-thirds of the value of gelatin chloride solutions of the same pH and the same concentration of originally isoelectric gelatin.

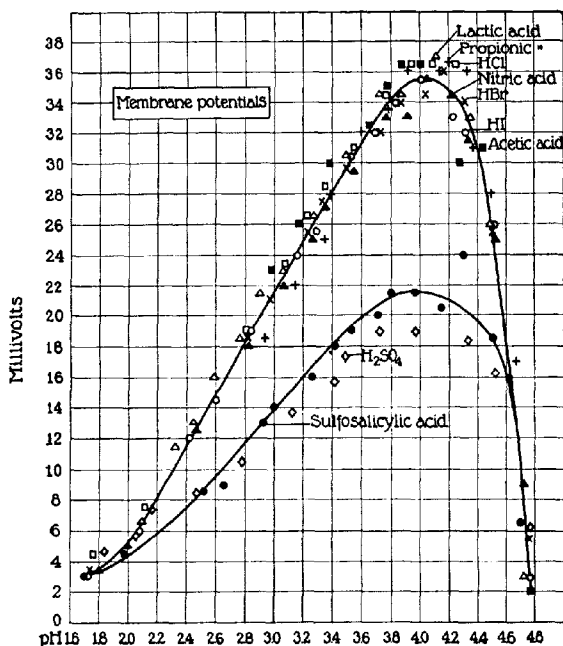


FIG. 60.—Proof that only the valency of an acid influences the membrane potentials of gelatin solutions. The ordinates are the membrane potentials in millivolts, abscissae the pH of gelatin solutions. The membrane potentials of the seven monobasic acids are practically identical, and so are the membrane potentials of the two strong dibasic acids.

The reader will notice that this relation holds for the same pH of the gelatin solution, but not for the same pH of the outside solution.

In order to test this idea, the influence of seven monobasic acids (HCl, HBr, HI, HNO₃, lactic, acetic, and propionic acids) was compared with the influence of two strong dibasic acids

(H_2SO_4 and sulphasalicylic acid) on the membrane potentials of solutions containing originally 1 gm. dry weight of isoelectric gelatin in 100 c.c. The P.D. was measured with the calomel electrodes as described. The observed P.D. are plotted in Fig.

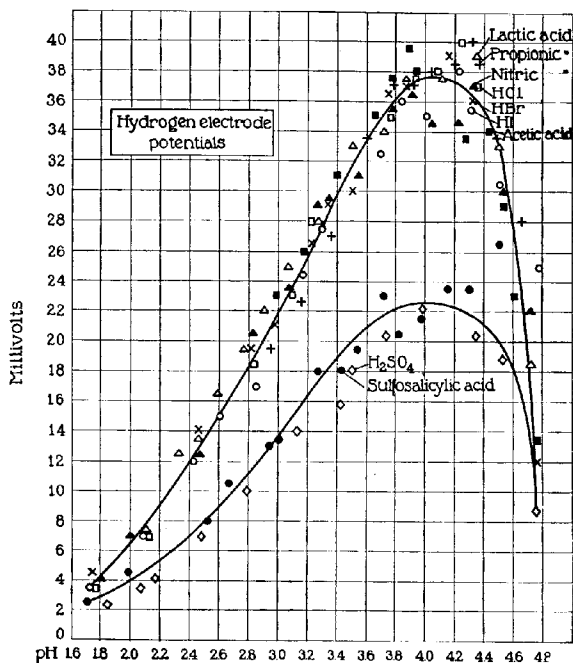


FIG. 61.—Proof that the influence of acids on the hydrogen electrode potentials of gelatin solutions is identical with that on the membrane potentials as shown in Fig. 60.

60 in terms of millivolts as ordinates over the pH of the gelatin solution after equilibrium was established (after 18 hours) at 24°C . The values for the influence of the seven monobasic acids on the membrane potentials of gelatin solutions fall within the limits of accuracy of the experiments on one curve; and so do

the values for the influence of the two strong dibasic acids, but the curves for the strong dibasic acids are considerably lower.

For the values of the two strong dibasic acids those of the sulphosalicylic acid were used, since a repetition of the experiment with H_2SO_4 gave the same values as those for sulphosalicylic acid.

The ratio of the values of the membrane potentials for dibasic and monobasic acids should always be about 0.66 for the same pH of the gelatin solution, and Table XV shows that this is correct within the limits of accuracy of the observations. It seems to the writer that this is a strong support of the idea that membrane potentials obey Donnan's theory.

TABLE XV.—MEMBRANE POTENTIALS FOR DIBASIC AND MONOBASIC ACIDS

pH	Dibasic acids, millivolts	Monobasic acids, millivolts	Ratio $\frac{\text{dibasic}}{\text{monobasic}}$
2.4	7.6	11.4	0.67
2.6	9.6	14.8	0.65
2.8	11.6	18.0	0.64
3.0	13.6	21.6	0.65
3.2	15.8	24.8	0.64
3.4	18.0	28.0	0.62
3.6	19.8	31.0	0.64
3.8	21.2	34.2	0.62
4.0	21.6	35.5	0.61
4.2	20.8	34.8	0.60
4.4	19.2	31.0	0.62

Further, in these experiments the hydrogen electrode potentials between the gelatin solution and the outside aqueous solution were measured. The values of the hydrogen electrode P.D. are plotted in Fig. 61. It is obvious that the curves for the membrane potentials (Fig. 60) are within the limits of experimental accuracy identical with the curves for the hydrogen electrode P.D. in Fig. 61.

For the sake of completeness we will give the effects of weak dibasic and tribasic acids, succinic, tartaric, and citric acids, on

the membrane potentials of gelatin solutions (containing 1 gm. dry weight of originally isoelectric gelatin in a 100-c.c. solution). All these acids dissociate as monobasic acids at pH 3.0 or below and Fig. 62 shows that for this range of pH the curves for the membrane potentials for the three acids coincide with that for HCl. When the pH rises, the second H begins to dissociate,

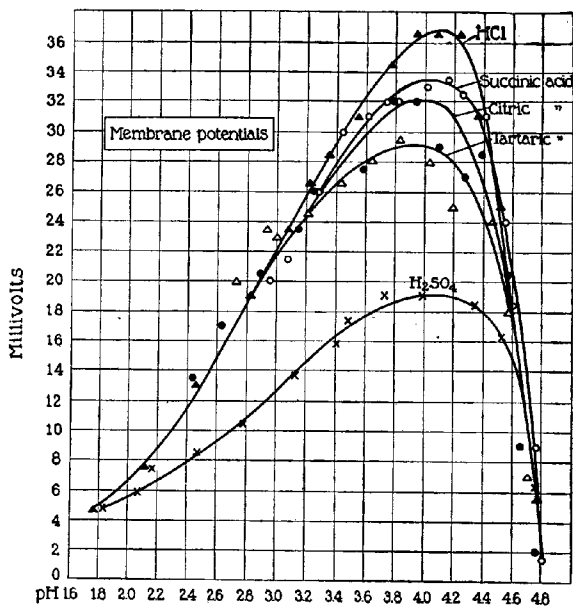


Fig. 62.—Influence of weak dibasic and tribasic acids on the membrane potentials of gelatin solutions.

and the more so the stronger the acid. For this reason the membrane P.D. curves for the weak dibasic or tribasic acids fall below that for HCl at a pH above 3.0, and the difference is the greater the stronger the acid. These curves might be used to determine at what pH and to what extent weak dibasic and tribasic acids begin to split off the second or third H ion with increasing pH. Figure 63 gives the hydrogen electrode potentials for

the same acids. The agreement between Figs. 62 and 63 is such that no doubt is left of the correctness of the Donnan equation for membrane potentials.

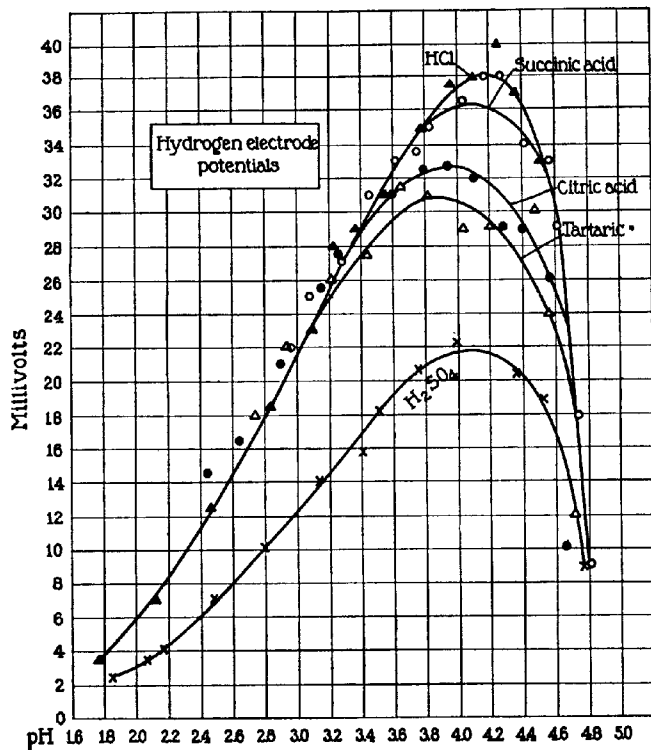


FIG. 63.—Influence of weak dibasic and tribasic acids on the hydrogen electrode potentials of gelatin solutions. Notice identity of curves in Figs. 62 and 63.

HYDROGEN ION AND CHLORINE ION POTENTIALS

If we write the equation for the equilibrium condition between gelatin chloride solution and water in the form

$$\frac{x}{y} = \frac{y+z}{x},$$

where x is the concentration of H and Cl ions in the outside solution, y the concentration of the H and Cl ions of the free HCl inside the gelatin chloride solution, and z the concentration of the Cl ions in combination with the gelatin, $\frac{x}{y}$ is the ratio of hydrogen ion concentration outside to the hydrogen ion concentration inside; and $\frac{y+z}{x}$ the ratio of the concentration of the chlorine ions inside to the chlorine ions outside. Since

$$\text{pH inside} = -\log y$$

and

$$\text{pH outside} = -\log x,$$

and hence

$$\log \frac{x}{y} = \text{pH inside minus pH outside}$$

and

$$\log \frac{y+z}{x} = \text{pCl outside minus pCl inside,}$$

it follows that

$$\text{pH inside minus pH outside} = \text{pCl outside minus pCl inside. (2)}$$

If Donnan's membrane equilibrium is the cause of the unequal distribution of crystalloidal ions on the opposite sides of the membrane, we must be able to show that equation (2) is actually fulfilled.

This consequence of Donnan's theory was put to a test and some of the experiments described in the preceding part of this chapter were selected for this purpose. Inside the collodion bags were 1 per cent solutions of gelatin chloride of different pH; the outside solutions consisted of water of originally the same pH as the gelatin solutions. After 18 hours, equilibrium was established between inside and outside solutions and the pCl as well as the pH was ascertained. The pCl was determined in two different ways in the two experiments; in one experiment it was determined with the calomel electrode, in the other it was determined in the gelatin chloride solution by titration with NaOH according to the method described in an earlier paper.¹

¹ LOEB, J., *J. Gen. Physiol.*, vol. 1, p. 363, 1918-19.

Both methods of determining the pCl led to the result that the value pCl outside minus pCl inside was for the same solution at the point of equilibrium equal to the value pH inside minus pH outside (within the limits of accuracy of the experiments). The pCl outside was identical with the pH outside, since the outside solution contained only free HCl. The values of pH were all determined with the hydrogen electrode (Table XVI).

TABLE XVI

Experiment 1. pCl determined by titration											
pH of gelatin chloride solution at equilibrium	4.13	3.69	3.30	3.10	2.92	2.78	2.46	2.26	2.01	1.76	
pH inside minus pH outside.....	0.56	0.58	0.50	0.49	0.44	0.44	0.33	0.23	0.15	0.10	
pCl outside minus pCl inside.....	0.48	0.51	0.59	0.44	0.44	0.38	0.35	0.22	0.15	0.11	

Experiment 2. pCl determined electrometrically											
pH of gelatin chloride solution at equilibrium	4.04	3.92	3.78	3.61	3.46	3.16	2.73	2.36	2.04	1.73	
pH inside minus pH outside.....	0.60	0.62	0.66	0.55	0.50	0.43	0.30	0.20	0.12	0.07	
pCl outside minus pCl inside.....	0.55	0.60	0.57	0.50	0.53	0.38	0.32	0.17	0.12	0.07	

This proves that the equation $x^2 = y(y + z)$ is the correct expression for the equilibrium between acid salts of proteins (with monovalent anion) and water, and that the Donnan equilibrium accounts for the membrane potentials observed. We wish to point out that we get about the same result whether we determine pCl by titration or potentiometrically. The agreement with the theory is the same in both cases, though the accuracy of the determination of pCl is less than that of pH.

This, then, leaves no doubt that the unequal distribution of crystalloidal ions on opposite sides of the membrane is governed by Donnan's formula for membrane equilibria.

ELIMINATION OF SOME INACCURACIES OF pH MEASUREMENTS
NEAR THE ISOELECTRIC POINT WITH THE AID OF BUFFER
SOLUTIONS

The agreement between the membrane potentials and hydrogen electrode potentials was good when the solution contained a neutral salt or when the hydrogen ion concentration of the solution was not too close to that of the isoelectric point; the agreement was, however, less satisfactory when the pH was near that of the isoelectric point of gelatin, *i.e.*, near pH 4.7, and no salts were present. The source of this disagreement seemed to lie in the inaccuracy in the measurement of the pH of the aqueous solution free from gelatin (the outside solution) at a pH between 4.0 and 7.0. If this surmise were correct, the disagreement in that region of hydrogen ion concentrations should be caused to disappear by the use of a buffer solution inside and outside.

One per cent solutions of isoelectric gelatin were made up in m/100 Na acetate solutions containing varying amounts of 1 M acetic acid, so that the pH of the gelatin solution varied (at the end of the experiment) between 4.65 (*i.e.*, practically isoelectric

TABLE XVII.—INFLUENCE OF pH ON P.D. OF SOLUTIONS OF GELATIN ACETATE IN THE PRESENCE OF BUFFER SOLUTION

Cubic centimeters 1 M acetic acid in 100-c.c. inside and outside solu- tions	1.0	1.5	2.0	3.0	4.0	6.0	10.0	15.0	20.0	30.0
Osmotic pressure, in mm. H ₂ O.....	21	31	34	43	47	62	83	95	103	108
pH inside.....	4.65	4.52	4.40	4.23	4.14	3.99	3.76	3.61	3.49	3.34
pH outside.....	4.65	4.50	4.37	4.19	4.09	3.92	3.69	3.53	3.39	3.23
pH inside minus pH outside.....	0	0.02	0.03	0.04	0.05	0.07	0.07	0.08	0.10	0.11
Hydrogen electrode P.D. (millivolts).....	0	1.0	2.0	2.5	3.0	4.0	4.0	5.0	5.5	7.0
Membrane P.D. (milli- volts).....	0.5	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.5	6.0

gelatin) and 3.34 (Table XVII). Collodion bags, of a content of about 50 c.c., were filled with these solutions of gelatin in buffer solutions, as described in Chap. VII. The bags were put into beakers containing 350 c.c. of identical solutions of $m/100$ Na acetate and 1 M acetic acid as those inside the bags, except that the 350-c.c. outside solutions contained no gelatin. The temperature was 24°C . After 24 hours the osmotic pressure, the membrane potentials, and the hydrogen electrode potentials were measured, and Table XVII shows that the two P.D. agree. The rest of the table needs no explanation.

Similar results were obtained in the case of solutions of edestin by Dr. Hitchcock.¹

THE P.D. OF Na GELATINATE

The Donnan theory demands that when a solution of Na gelatinate contained in a collodion bag is in equilibrium with an aqueous solution free from gelatin, free NaOH should be forced from the inside gelatin solution through the membrane into the outside aqueous solution free from gelatin. As a result, the pH inside will now be less than pH outside, and the value pH inside minus pH outside will be negative for Na gelatinate, while it was positive for gelatin chloride. If the Donnan equilibrium determines the P.D. (as it does), the sign of charge of Na gelatinate must be the reverse from what it was for gelatin chloride. This is, indeed, the case and the turning point lies, as was expected, at the isoelectric point.

The experiments with Na gelatinate demand greater precautions than those with gelatin chloride. It is necessary to prevent the CO_2 of the air from diffusing into the alkaline solutions and therefore the outside solution was put into stoppered bottles connected with the outside air by glass tubes filled with soda lime.

Collodion bags of a volume of about 50 c.c. were filled with solutions of Na gelatinate containing 1 gm. of originally isoelectric gelatin and varying amounts of 0.1 N NaOH in a 100-c.c. solution. The collodion bags were dipped into flasks containing 500 c.c. of aqueous solutions of NaOH of various concentrations and free from gelatin. The flasks were stoppered, communi-

¹ HITCHCOCK, D. I., *J. Gen. Physiol.*, vol. 4, p. 597, 1921-22.

TABLE XVIII.—1 PER CENT Na GELATINATE

Cubic centimeters 0.1 N NaOH added to 1 gm. gelatin in 100 c.c.	0	1	2	3	4	5	6	8	10	12.5	15	20
Concentrations of NaOH of outside solution	0	0	0	N/25,600	N/12,800	N/6,400	N/3,200	N/1,600	N/800	N/400	N/200	N/100
Osmotic pressure, millimeters	26	164	265	353	375	385	366	335	340	265	192	150
pH inside	5.02	5.40	5.76	6.64	7.15	9.02	9.68	10.16	10.45	...	11.30	11.58
pH outside	5.60	5.82	5.92	6.37	7.70	9.50	9.96	10.60	10.85	...	11.46	11.70
pH inside minus pH outside	- 0.58	- 0.42	- 0.16	+ 0.27	- 0.55	- 0.48	- 0.28	- 0.44	- 0.40	...	- 0.16	- 0.12
Hydrogen electrode P.D. (milli- volts)	-34.0	-24.5	- 9.4	+15.8	-32.0	-28.0	-16.5	-25.7	-23.4	...	- 9.4	- 7.0
Membrane P.D. (millivolts)	- 3.5	-19.5	-18.0	-37.5	-37.5	-36.0	-30.0	-22.0	-19.5	...	-10.0	- 7.0

cating with the air only through tubes filled with soda lime, as stated. The collodion bags containing the gelatin were closed by rubber stoppers perforated by glass tubes which served as manometers. The experiment lasted 6 hours at a temperature of 24°C. The results of the experiments are given in Table XVIII. The upper horizontal row gives the number of cubic centimeters of 0.1 *N* NaOH originally in 100 c.c. of the gelatin solution; the second row gives the original concentration of NaOH in the outside aqueous solution free from gelatin; the third row gives the osmotic pressure in mm. H₂O after 6 hours. The next row gives the pH inside and the following row the pH outside after the experiment was finished (*i.e.*, after 20 hours), and the sixth row gives the difference pH inside minus pH outside. The reader will notice that this difference is always negative with one exception, which is obviously an error. The last two rows give the hydrogen electrode potentials and the membrane potentials.

The determination of the hydrogen electrode P.D. between the inside and outside of the Na gelatin solution suffers from a serious source of error, which will be recognized by a glance at the nature of the titration curve for gelatin with NaOH (Chap. IV). This curve runs almost parallel with the axis of abscissæ for a pH between 6.0 and 8.5, which means that a trace of NaOH can shift the pH one or two whole units; in other words, the difference between membrane potentials and hydrogen electrode potentials may be as much as 50 millivolts or more in this region. Since nearer the isoelectric point the pH measurements are also unreliable, we can only expect an agreement between the two kinds of P.D. when the pH is 8.0 or more. Table XVIII shows that this is true.

It is obvious that there is no quantitative agreement between membrane potentials and hydrogen electrode potentials near the isoelectric point. As soon as the pH is above 7.0 the agreement between the two kinds of P.D. becomes better, so that we are entitled to say that the difference of potential between a Na gelatin solution and an outside solution at or near equilibrium is due to the Donnan equilibrium which forces the expulsion of NaOH from the inside into the outside solution. As a consequence the pH inside becomes lower than the pH outside.

THE INFLUENCE OF NEUTRAL SALTS ON THE P.D. OF GELATIN CHLORIDE SOLUTIONS

It was shown in Chap. VIII that the addition of neutral salts to solutions of protein salts depresses the osmotic pressure or viscosity of these solutions, and that the addition of neutral salts to a gel depresses the swelling of the latter (except when the solutions and gels are at the isoelectric point). It was of interest to find out whether or not the addition of a salt to a protein solution depresses also the P.D. across a collodion membrane, and whether this is also due to a depression of the value of pH inside minus pH outside. It was possible to show that this is true.

Gelatin chloride solutions containing 1 gm. of originally isoelectric gelatin in a 100-c.c. solution and having a pH of 3.5 were made up in different concentrations of NaNO_3 in water, the concentrations of NaNO_3 varying from $m/4,096$ to $m/32$, all possessing a pH of 3.5. These mixtures were put into collodion bags and the bags were put into HCl solutions of pH 3.0 made up in different concentrations of NaNO_3 , also of pH 3.0. These outside solutions contained no gelatin. The collodion bags were put into these outside solutions free from gelatin in such a way that the concentration of the NaNO_3 solution inside the collodion bag was always the same as outside.

When the P.D. across the collodion membrane was measured after 18 hours (after equilibrium was established), it was found that it was diminished upon the addition of neutral salt, and the more the higher the concentration of the salt (Fig. 64, p. 205). This shows that the addition of neutral salt to a protein solution has a similar depressing effect on the P.D. as on the osmotic pressure, swelling, and viscosity of the protein solutions.

The next fact of interest was that the values of the hydrogen electrode potentials diminish in a parallel way with the membrane potentials and that both P.D. agree remarkably well (Table XIX).

We have seen in Chap. VIII that the addition of a salt with bivalent anion, *e.g.*, Na_2SO_4 , to a gelatin chloride solution has a much greater depressing effect on the osmotic pressure, viscosity, etc. of the solution than the addition of a salt with monovalent anion, namely, NaNO_3 . It can be shown that the addition of Na_2SO_4 also has a greater depressing effect on the membrane

TABLE XIX.—DEPRESSING EFFECT OF NEUTRAL SALTS ON P.D. OF GELATIN CHLORIDE SOLUTIONS

Concentration of NaNO ₃									
0	m/4,096	m/2,048	m/1,024	m/512	m/256	m/128	m/64	m/32	
pH inside.....	3.58	3.56	3.51	3.46	3.41	3.36	3.32	3.29	3.25
pH outside.....	3.05	3.08	3.10	3.11	3.14	3.17	3.20	3.22	3.24
pH inside minus pH outside.....	0.53	0.48	0.41	0.35	0.27	0.19	0.12	0.07	0.01
Hydrogen electrode P.D. (millivolts).....	+31.2	+28.3	+24.0	+20.7	+16.0	+11.2	+7.0	+4.1	0
Membrane P.D. (millivolts).....	+31.0	+28.0	+24.0	+22.0	+16.0	+12.0	+7.0	+4.0	0

TABLE XX.—DEPRESSING INFLUENCE OF Na_2SO_4 ON THE P.D. OF GELATIN CHLORIDE SOLUTIONS

	Concentration of Na_2SO_4									
	0	m/4,096	m/2,048	m/1,024	m/512	m/256	m/128	m/64	m/32	m/16
pH inside.....	3.54	3.41	3.35	3.32	3.29	3.30	3.33	3.33	3.41	3.41
pH outside.....	3.07	3.12	3.14	3.17	3.20	3.24	3.30	3.35	3.38	3.38
pH inside minus pH outside.....	0.47	0.29	0.21	0.15	0.09	0.06	0.03	0.03	0.03	0.01
Hydrogen electrode P.D. (millivolts).....	+27.6	+17.0	+12.3	+8.8	+5.3	+3.5	+1.7	+1.7	+1.7	+0.6
Membrane P.D. (millivolts).....	+26.5	+18.6	+12.5	+8.4	+4.7	+3.4	+1.5	0.0	0.0	0.0

TABLE XXI.—DEPRESSING INFLUENCE OF CaCl_2 ON THE P.D. OF GELATIN CHLORIDE SOLUTIONS

	Concentration of CaCl_2										
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
pH inside.....	3.55	3.45	3.41	3.36	3.30	3.28	3.26	3.25	3.25	3.25	3.22
pH outside.....	3.05	3.06	3.09	3.12	3.15	3.17	3.20	3.22	3.24	3.24	3.22
pH inside minus pH outside.....	0.50	0.39	0.32	0.24	0.15	0.11	0.06	0.03	0.01	0.01	0.0
Hydrogen electrode P.D. (millivolts).....	+29.5	+23.0	+18.9	+14.1	+8.8	+6.5	+3.5	+1.8	+0.6	+0.6	0.0
Membrane P.D. (millivolts).....	+28.6	+23.4	+19.2	+14.5	+9.1	+5.7	+3.1	+1.8	+1.1	+0.5	+0.5

potentials of a gelatin chloride solution than has a NaNO_3 solution of the same molecular concentration (Table XX).

We will consider as a third case the influence of CaCl_2 on the membrane P.D. of a gelatin chloride solution. It has been shown that the depressing effect of CaCl_2 on the osmotic pressure of a gelatin chloride solution is twice as great as that of an equimolecular concentration of NaCl . Table XXI shows that the depressing influence of CaCl_2 on the P.D. is about twice as great as that of NaNO_3 . The agreement between the membrane potentials and the hydrogen electrode potentials is excellent.

It is of importance that the depressing effect of salts on the P.D. can be derived from the Donnan theory. To show this we must remember that the P.D. is expressed by the following term:

$$\text{P.D.} = \frac{58}{2} \log \left(1 + \frac{z}{y} \right) \text{ millivolts.}$$

When we add NaCl to a gelatin chloride solution we increase the concentration of the chlorine ions not in combination with gelatin, *i.e.*, y , while the concentration z of the Cl ions in combination with the gelatin remains the same, provided the pH remains the same (neglecting the diminution of ionization of gelatin chloride). Hence, the P.D. must become the smaller the greater y , and with steadily increasing y and constant z the value of $1 + \frac{z}{y}$ must approach 1; *i.e.*, the addition of enough salt must depress the P.D. to zero, which is actually the case.

This is also true when we add another salt, *e.g.*, NaNO_3 , to a gelatin chloride solution. In this case we may assume that gelatin nitrate is formed.

The depressing effect of the addition of NaCl to gelatin chloride solution on the P.D. can be derived from the values of pH inside minus pH outside. The question arises: Why is it correct to neglect the influence of the Na ion? The writer did not give any reason for this, but Dr. J. A. Wilson was kind enough to point out in a letter the mathematical proof justifying the writer's procedure in the following way:

The true expression of the P.D. of a gelatin chloride solution in the presence of NaCl is

$$\text{P.D.} = \frac{RT}{F} \log \frac{\left[\text{H}^+ \right]_{\text{outside}} + \left[\text{Na}^+ \right]_{\text{outside}}}{\left[\text{H}^+ \right]_{\text{inside}} + \left[\text{Na}^+ \right]_{\text{inside}}}$$

Let the system contain the positive ions A, B, C , etc., and the negative ions M, N, O , etc., whose concentrations in the outside solution are, a, b, c, m, n, o , etc., and in the inside solution, a', b' , etc. From the published work of Procter and Wilson it is evident that the product of concentration of any pair of oppositely charged ions is equal in both phases. The following equations are evident:

$$\begin{aligned} a \times m &= a' \times m' \\ b \times n &= b' \times n' \\ (a + b + c + \dots)m &= (a' + b' + c' + \dots)m' \\ (a + b + c + \dots)(m + n + o + \dots) &= \\ &= (a' + b' + c' + \dots)(m' + n' + o' + \dots) \end{aligned}$$

whence

$$\frac{a}{a'} = \frac{b}{b'} = \frac{c}{c'} = \frac{a + b + c + \dots}{a' + b' + c' + \dots}$$

It is, therefore, immaterial which ion is singled out for the calculation of the P.D. on the basis of the Donnan effect. For the sake of the accuracy of measurement the hydrogen ion was selected.

It is perhaps worth while to point out that the agreement between membrane potentials and hydrogen electrode potentials is better in the experiments with salts than in the experiments without salts, especially near the isoelectric point.

These experiments, then, leave no doubt that the depressing effect of salts on the membrane potentials is due to a diminution in the difference in the concentration of crystalloidal ions on opposite sides of the membrane and that this difference can again be calculated from Donnan's formula.

THE INFLUENCE OF THE SIGN OF CHARGE

The fact that the P.D. of a protein-acid salt solution is a function of the term, $\log \left(1 + \frac{z}{y} \right)$, where z is the concentration of the anion in combination with the protein ions and y the concentration of the anion of the free acid, explains a phenomenon which is fundamental in colloidal behavior, namely, that whenever a salt depresses any physical property of a protein (or a colloidal solution in general) this action is due to that ion of the salt which has the opposite sign of charge to that of the protein ion. That this is true for the influence of salts on viscosity,

osmotic pressure, and swelling has been discussed in Chap. VIII. In all these cases the efficiency of the salt increases with the valency of the efficient ion of the salt. These rules are a consequence of the Donnan equilibrium. The term, $\log \left(1 + \frac{z}{y}\right)$, derived from the equilibrium equation makes the P.D. a function of z and y , *i.e.*, that ion which has the opposite sign of charge to that of the protein ion.

It follows from this that NaCl, CaCl₂, and LaCl₃ should have the same depressing effect on the membrane potentials of gelatin chloride solutions if they possess equal concentrations of Cl ions. This was found to be correct. One gram of gelatin chloride of pH 3.0 was dissolved in 100 c.c. of solutions of various salts all brought to pH 3.0 through the addition of HCl. Collodion bags were filled with these solutions of gelatin chloride in different salts, and each bag was dipped into 350 c.c. of an aqueous solution of the same concentration of the same salt and the same pH as that inside the collodion bag, but without gelatin. Water diffused into the collodion bag until osmotic equilibrium was established, and the next day the final osmotic pressure and the P.D. between gelatin solution and outside aqueous solution were measured. The solutions of the three salts were prepared in such a way as to have the same concentration of Cl. In Fig. 64 are plotted the P.D. as ordinates over the concentration of the Cl ions as abscissæ. It is obvious that the influence of NaCl, CaCl₂, and LaCl₃ on the P.D. is identical for the same concentration of Cl. This proves that only the Cl ion of these salts influences the P.D. of a gelatin chloride solution of pH 3.0 and that the La ion or the other cations do not increase the P.D. of the solution.

It follows, furthermore, that all salts with an anion of the same valency should depress the membrane potentials of a gelatin chloride solution to the same extent. This was found to be true. The pH of the gelatin chloride solution in these experiments was 3.8 and care was taken that this pH was not altered by the addition of salt. For this purpose three stock solutions, all of pH 3.8, were prepared. First, solutions of gelatin chloride of pH 3.8 containing 2 gm. dry weight of originally isoelectric gelatin and 8 c.c. of 0.1 N HCl in a 100-c.c. solution; second, $m/2$ solutions of different salts brought to a pH of 3.8 by the addition of HCl;

and third, distilled water brought also to the pH of 3.8 by the addition of HCl. By successive dilution of the $M/2$ salt solutions of pH 3.8 with distilled water of pH 3.8, series of salt solutions of different degree of concentration, but all of pH 3.8, were prepared. Fifty cubic centimeters of the 2 per cent solutions of gelatin chloride of pH 3.8 and 50 c.c. of the salt solutions of pH 3.8

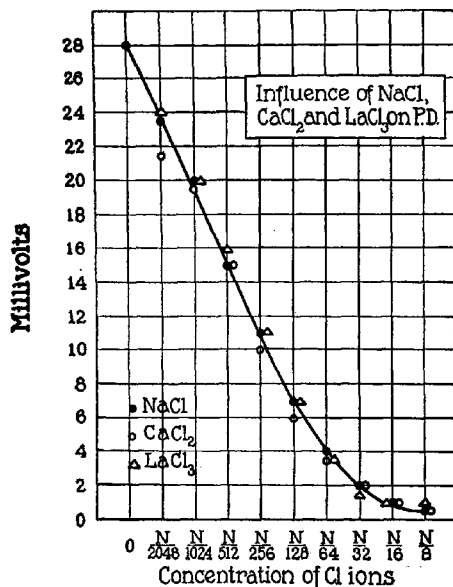


FIG. 64.—Depressing influence of NaCl, CaCl₂, and LaCl₃ on the membrane potentials between a 1 per cent solution of gelatin chloride of pH about 3.0 and aqueous solutions of the salts originally of the same pH, the two solutions being separated by collodion membranes. Ordinates are the P.D. in Millivolts, abscissæ the concentrations of Cl ions of the salts. The depressing effect of the three salts is the same for the same concentration of Cl ions, proving that the cations do not influence the P.D. of gelatin chloride solutions.

were then mixed, and 1 per cent solutions of gelatin chloride in salt solutions of different concentrations but all of pH 3.8 were obtained.

Collodion bags of about 50-c.c. volume were filled with such solutions (closed with stoppers perforated by manometer tubes

as described) and the collodion bags were submerged in beakers containing each 350 c.c. of the same salt solution as that inside the collodion bags and all of pH 3.8, but without protein. The experiments lasted for 18 to 24 hours at 24°C. At that time osmotic equilibrium was reached, the osmotic pressure was read,

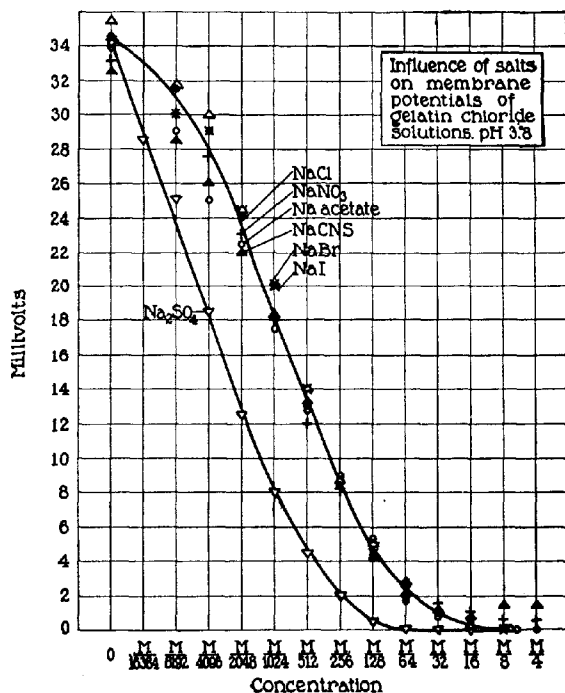


FIG. 65.—All salts with monovalent anions have within the limits of experimental accuracy the same depressing effect on the membrane potentials of gelatin chloride solutions at pH 3.8, while the depressing effect of Na_2SO_4 is much greater.

and the membrane potentials between the protein solution inside the collodion bag and the outside aqueous solution free from protein were measured with a pair of indifferent calomel electrodes (in saturated KCl) as described.

Figure 65 represents the effect of six different salts with monovalent anions, NaCl, NaBr, NaI, NaNO₃, NaCNS, and Na acetate, and one salt with divalent anion, Na₂SO₄. The abscissæ are the concentrations of the salts and the ordinates are the observed membrane potentials in millivolts. When no salt was added, *i.e.*, when the concentration was zero, the values vary within about 3 to 4 millivolts, due to the limits of experimental accuracy. This variation was, of course, also found when salts were added. The curves in Fig. 65 show that the only variation between the effects of the six different salts with monovalent anion is due to the limits of accuracy of the measurements, and that, if this is taken into consideration, it is found that the effects of the six salts on membrane potentials lie all on one curve. The curve for Na₂SO₄ is, however, considerably lower than the curve for the six salts with monovalent anions, namely, a little less than $\frac{2}{3}$. The curves in Fig. 65, therefore, show that the six salts, NaCl, NaBr, NaI, NaNO₃, NaCNS, and Na acetate, have (within the limits of accuracy of measurements) the same effect on the membrane potentials, while SO₄ depresses the value to about $\frac{2}{3}$. These experiments prove that all the salts with an anion of the same valency have the same depressing effect on the membrane potentials of gelatin chloride solutions.

THE INFLUENCE OF THE CONCENTRATION OF PROTEIN ON THE MEMBRANE POTENTIALS

While the addition of neutral salt depresses the membrane potentials of protein solutions across a membrane (as it depresses all the other properties), the addition of protein has the opposite effect, increasing the P.D. (as it increases also the other properties). This influence of the concentration of the protein follows mathematically from the equilibrium equation. Since

$$\text{P.D.} = \frac{58}{2} \log \left(1 + \frac{z}{y} \right) \text{ millivolts,}$$

it is obvious that if y remains constant (*i.e.*, if no salt is present and the pH remains the same) while z increases as a consequence of the increase of the concentration of protein, the P.D. must rise with the concentration, and this was found to be the case.

TABLE XXII

	Per cent of gelatin in solution											
	2	2	1½	1½	1	1	¾	¾	½	½	¼	¼
pH inside.....	3.64	3.66	3.60	3.60	3.65	3.66	3.60	3.60	3.61	3.62	3.57	3.47
pH outside.....	3.02	3.02	3.02	3.01	3.12	3.11	3.14	3.12	3.21	3.19	3.25	3.29
pH inside minus pH outside.....	0.62	0.64	0.58	0.59	0.53	0.55	0.46	0.48	0.40	0.43	0.32	0.18
Hydrogen electrode P. D. (millivolts).....	+36.6	+38.7	+34.2	+34.8	+31.3	+32.4	+27.2	+28.3	+23.6	+25.3	+18.8	+10.6
Membrane P. D. (millivolts).....	+34.0	+36.5	+32.3	+34.0	+31.8	+32.3	+28.6	+28.6	+22.7	+22.7	+19.0	+13.0

Collodion bags, connected with glass manometers in the way described, containing 50 c.c. of different concentrations of originally isoelectric gelatin varying from 0.125 to 2 per cent and containing enough H_2PO_4 to bring the gelatin solution to a pH of 3.5 were put into beakers containing 350 c.c. of H_3PO_4 solution of pH 3.5. In order to prevent dilution of the protein solution through osmosis, the glass manometers were filled at the beginning of the experiment with the same gelatin phosphate solution as that contained in the collodion bag, to the height which the osmotic pressure measured in preceding experiments amounted to. After about 20 hours the hydrogen electrode potentials and the membrane potentials across the membrane were measured. Some of the experiments were made in duplicate (Table XXII).

It is obvious, first, that the membrane potentials increase with the concentration of gelatin, and second, that the increase agrees quantitatively with the increase in the hydrogen electrode potentials.

THE P.D. OF SOLUTIONS OF CRYSTALLINE EGG ALBUMIN

The experiments mentioned thus far had almost all been made with gelatin. It was of importance to determine whether or not these results could be confirmed with crystalline egg albumin. This was found to be the case, and the experiments on the membrane potentials of the solutions of the chloride of crystalline egg albumin showed a perfect quantitative agreement with the theory.

Collodion bags of about 50-c.c. volume were filled with a solution of 1 per cent crystalline egg albumin containing varying amounts of 0.1 N HCl, and the bags were put, as usual, into beakers containing 350 c.c. of HCl solutions of different concentration but free from albumin. The first two horizontal rows of Table XXIII give the amount of 0.1 N HCl in each solution. The experiments were carried out at a temperature of 24°C., and after 22 hours the osmotic pressure, P.D., and pH of inside (albumin) solution and pH of the outside solution were measured. The albumin used was not isoelectric, but since it had been prepared after Sørensen's method it was probably partly ammonium albuminate, with a pH of near 6.0. The table shows that the membrane potentials and the hydrogen electrode

TABLE XXIII.—ONE PER CENT ALBUMIN CHLORIDE

	0	1	2	3	4	5	6	7	8	10	15	20	30	40
Cubic centimeters 0.1 N HCl in a 100-c.c. solution containing 1 gm. originally isoelectric albumin.....	0	0.1	0.3	0.5	1	1.5	2.1	2.8	4	7.1	16.4	32	60	80
Cubic centimeters 0.1 N HCl in a 350-c.c. outside solution.....	0	0.1	0.3	0.5	1	1.5	2.1	2.8	4	7.1	16.4	32	60	80
Osmotic pressure in millimeters.....	155	100	52	114	178	205	214	219	218	180	138	100	81	74
pH inside.....	5.80	5.40	4.70	4.30	4.00	3.75	3.64	3.42	3.24	3.00	2.53	2.20	1.80	1.73
pH outside.....	6.14	5.64	4.67	4.06	3.65	3.38	3.22	3.07	2.91	2.71	2.37	2.10	1.82	1.70
pH inside minus pH outside.....	- 0.34	- 0.24	+0.03	+ 0.24	+ 0.35	+ 0.37	+ 0.42	+0.35	+ 0.33	+ 0.29	+ 0.16	+ 0.10	+0.07	+0.03
Hydrogen electrode P.D. (millivolts).....	-20.0	-14.0	+2.0	+14.0	+20.6	+22.4	+25.5	+21.0	+20.0	+17.5	+ 9.4	+ 6.0	+4.0	+2.0
Membrane P.D. (millivolts).....	-24.0	-16.0	+3.0	+11.5	+19.0	+19.5	+20.5	+19.5	+18.5	+16.0	+11.0	+10.0	+4.0	+3.5

TABLE XXIV.—INFLUENCE OF SALT ON P.D. OF ALBUMIN CHLORIDE SOLUTION

	Concentration of NaCl									
	0	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
Osmotic pressure, millimeters.	210	181	156	131	107	87	73	61	54	45
pH inside.	3.35	3.32	3.32	3.27	3.25	3.20	3.19	3.22	3.21	3.22
pH outside.	3.04	3.04	3.07	3.10	3.11	3.13	3.14	3.18	3.21	3.23
pH inside minus pH outside.	0.31	0.28	0.25	0.17	0.14	0.07	0.05	0.04	0.00	-0.01
Hydrogen electrode P.D. (millivolts)	+18.0	+16.2	+14.5	+10.0	+8.0	+4.1	+2.9	+2.3	0.0	-0.5
Membrane P.D. (millivolts)	+18.5	+15.5	+13.5	+10.0	+7.5	+5.0	+3.0	+1.5	+1.0	+0.5

potentials agree closely (especially on the acid side of the isoelectric point); that the P.D. is a minimum near pH 4.70 of the albumin (*i.e.*, near the isoelectric point, which is at pH 4.8), and that the albumin is positively charged on the acid and negatively charged on the alkaline side of the isoelectric point. This is again in harmony with what we should expect on the basis of the Donnan equilibrium.

The next problem was to determine the influence of the addition of a neutral salt to a solution of the chloride of crystalline egg albumin. A 1 per cent solution of crystalline egg albumin containing 7 c.c. of 0.1 *N* HCl in 100 c.c. was made up in various concentrations of NaCl. The collodion bags containing these albumin chloride-NaCl mixtures were dipped into beakers containing 350 c.c. the same concentration of NaCl as that of the albumin solution, and all made up in *N*/1,000 HCl. The experiment was carried out at 24°C. and the measurements were made after 22 hours.

Table XXIV gives the results, which show again an excellent agreement between membrane potentials and hydrogen electrode potentials.

MEMBRANE POTENTIALS OF CASEIN CHLORIDE SOLUTIONS

It can also be shown that in the case of casein chloride solutions the distribution of crystalloidal ions on opposite sides of the membrane is determined by the Donnan equilibrium.

Thus, 4, 3, 2, 1, and 0.5 per cent solutions of isoelectric casein were brought to about pH 2.5 by adding HCl and put into collodion bags as described, and each bag was immersed in 350 c.c. of HCl solution of initial pH 2.3. After 18 hours the potential differences between the casein solution (inside solution) and the outside aqueous solution free from casein were determined with the indifferent calomel electrodes and afterwards the pH of the inside and outside solutions were measured with the hydrogen electrode. Table XXV gives the results of the measurements.

The second and the third rows give the pH of the inside and outside solutions as measured with the hydrogen electrode after osmotic equilibrium was established. The fourth row gives the values pH inside minus pH outside and the fifth row the hydrogen electrode potentials. These latter values should agree with the

TABLE XXV.—AGREEMENT BETWEEN MEMBRANE POTENTIALS AND HYDROGEN ELECTRODE POTENTIALS

1. Casein chloride, per cent. . .	4.0	3.0	2.0	1.0	0.5	0.25
2. pH of inside solution at equilibrium.	2.595	2.595	2.580	2.53	2.46	2.46
3. pH of outside solution at equilibrium.	2.230	2.270	2.305	2.34	2.36	2.39
4. pH inside minus pH outside. .	0.365	0.325	0.275	0.19	0.10	0.07
5. Hydrogen electrode P.D. (millivolts).	21.5	19.2	16.2	11.2	5.9	4.1
6. Membrane P.D. (millivolts). .	20.0	18.0	15.0	10.8	7.2	3.1

values for membrane potentials between inside and outside solutions obtained with the aid of indifferent electrodes, and a comparison of the fifth and sixth rows of Table XXV shows that the agreement is good. The agreement between the values in the last two rows of Table XXV leaves little doubt that the membrane potentials are, indeed, the result of Donnan's membrane equilibrium.

The observations also confirm once more the fact that, for the same pH of the solution of the protein salt, the membrane potential increases with the concentration of the protein, as the theory of membrane equilibria demands.

Hitchcock has shown that the Donnan equilibrium determines also the unequal distribution of crystalloidal ions in the case of solutions of edestin and globulin.¹

CONCLUDING REMARKS

We have seen that the value of the membrane potential is equal to $29 \log \left(1 + \frac{z}{y}\right)$ millivolts. Since the addition of a non-electrolyte cannot affect the value of $\frac{z}{y}$, it follows that the addition of a non-electrolyte like cane sugar to a gelatin chloride solution cannot influence the P.D. This was confirmed experimentally.

¹ HITCHCOCK, D. I., *J. Gen. Physiol.*, vol. 4, p. 597, 1921-22; vol. 5, p. 35, 1922-23.

The membrane potential may become zero in two ways: At the isoelectric point z is zero and the value $29 \log \left(1 + \frac{z}{y}\right)$ becomes zero.

On the other hand, when a salt is added in sufficiently high concentration, the membrane potential becomes zero, because in the term $29 \log \left(1 + \frac{z}{y}\right) y$ becomes very large so that $\frac{z}{y}$ approaches zero.

It is often stated that the addition of salt brings proteins to the isoelectric point. This is not correct, since the isoelectric point is a constitutional property of a protein which only occurs at a definite hydrogen ion concentration. Salts annihilate the P.D. but do not bring the protein to the isoelectric point. Protein solutions or gels may be uncharged without being at the isoelectric point.

These data furnish the proof which is fundamental for the interpretation of the influence of electrolytes on the colloidal behavior of proteins—especially their osmotic pressure, namely, that the difference in the distribution of crystalloidal ions between protein solution inside a collodion bag and aqueous solution free from protein is determined by the Donnan equilibrium, and that it can be calculated on the basis of the equation for this equilibrium. Other facts concerning the membrane potentials will be discussed in later Chapters of this volume.

CHAPTER XII

OSMOTIC PRESSURE¹

THE INFLUENCE OF THE HYDROGEN ION CONCENTRATION

We are now in a position to explain the influence of electrolytes on the osmotic pressure of protein solutions, as expressed in Fig. 57. We will begin with a discussion of the influence of acid on the osmotic pressure of 1 per cent solutions of originally isoelectric gelatin. Solutions containing 1 gm. dry weight of originally isoelectric gelatin in 100 c.c. and containing different quantities of 0.1 N acid were prepared. Collodion bags, cast in the form of Erlenmeyer flasks of 50-c.c. volume, were filled with these solutions and put into beakers containing 350 c.c. of H₂O. In order to accelerate the establishment of the equilibrium between inside and outside solution, a certain amount of acid was added to the outside water (*e.g.*, HCl in the experiments with gelatin chloride, H₃PO₄ in the case of gelatin phosphate, etc.). Each collodion bag was closed with a rubber stopper perforated by a glass tube serving as a manometer. All this was described more in detail in Chap. VII.

Figure 66 shows the influence of HCl, H₃PO₄, and H₂SO₄ on the osmotic pressure of a 1 per cent solution of originally isoelectric gelatin. The abscissæ are the pH of the gelatin solution at osmotic equilibrium, and the ordinates are the osmotic pressures in terms of millimeter H₂O. The osmotic pressure is a minimum at the isoelectric point, rises with the addition of acid until a maximum is reached at a pH of about 3.4 of the gelatin solution, and then falls again with the addition of more acid.

Before we have a right to indulge in speculations concerning the cause of the influence of acid we must realize that these curves of observed osmotic pressure are not exclusively the expression of the osmotic pressure due to the protein molecules and protein

¹ LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 691, 1920-21; vol. 4, pp. 741, 769, 1921-22; *J. Am. Chem. Soc.*, vol. 44, p. 1930, 1922.

ions alone, but are also the result of the demonstrable unequal concentrations of the crystalloidal ions on the opposite sides of the membrane, caused by the establishment of a Donnan equilibrium. In other words, the observed osmotic pressure of a protein solution needs a correction due to the Donnan equilibrium, and it is our purpose to calculate the value of this correction.

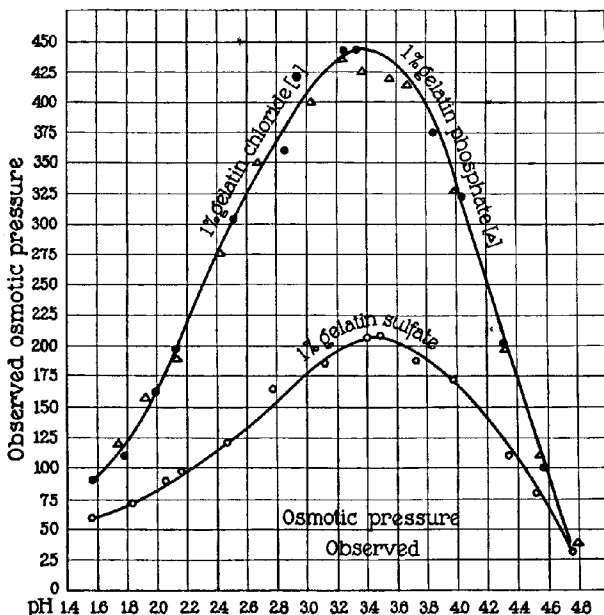


FIG. 66.—Observed curves representing the influence of pH and valency of anion on osmotic pressure of solutions of gelatin-acid salts containing 1 gm. of originally isoelectric gelatin in a 100-c.c. solution. The curves for gelatin chloride and gelatin phosphate are identical, since the anions, Cl and H_2PO_4 , of these two gelatin salts are monovalent. The curve for gelatin sulphate is less than half as high as the curve for the two other salts because the anion of gelatin sulphate is bivalent. Both curves rise from the isoelectric point at 4.7 to a maximum at pH about 3.4 or 3.5, and then drop rapidly again.

We begin with the curve expressing the influence of HCl on the osmotic pressure of a 1 per cent solution of originally isoelectric gelatin, and we consider the distribution of ions inside the protein solution and in the aqueous solution outside the collodion

bag containing the protein solution at osmotic equilibrium. We also assume complete electrolytic dissociation of all the electrolytes, gelatin chloride as well as HCl. Let a be the molar concentration of the protein molecules and ions, let z be the molar concentration of the Cl ions in combination with the ionized protein, let y be the molar concentration of the hydrogen ions of the free HCl inside the protein solution; the molar concentration of the Cl ions of this HCl is also y . In that case the osmotic pressure of the protein solution is determined by

$$a + 2y + z.$$

From this must be deducted the osmotic pressure of the HCl of the outside aqueous solution. If x is the molar concentration of the H ions of the outside solution, it is also the molar concentration of the Cl ions. Hence, the observed osmotic pressure of a protein solution is determined by the following molar concentration:

$$a + 2y + z - 2x.$$

Figure 66 shows that this value varies with the pH of the protein solution (*i.e.*, y). In order to arrive at a theory concerning the influence of HCl on the osmotic pressure of protein solutions, it is necessary to calculate the value of $2y + z - 2x$, to determine its theoretical osmotic pressure according to van't Hoff's theory, and to deduct this latter value from the observed osmotic pressure of the protein solution. We will call the osmotic pressure determined by the molar concentration, $2y + z - 2x$, the "Donnan correction."¹ In this term, y and x can be calculated from the measurements of the pH, pH inside being $-\log y$, and pH outside being $-\log x$. z can be calculated from x and y with the aid of the Donnan equation (1),

$$z = \frac{(x + y)(x - y)}{y},$$

since we now know from the preceding chapter that x and y are determined by the Donnan equilibrium. If the value of $2y + z - 2x$ is calculated for different pH of a gelatin chloride solution (of the same concentration of originally isoelectric gelatin, which in this case was 1 per cent), and if from this value is calculated

¹ For the sake of brevity we will occasionally refer to the term $2y + z - 2x$ as the "Donnan correction."

the osmotic pressure due to this excess of the molar concentration of crystalloidal ions inside the protein solution over that outside, on the basis of van't Hoff's theory, it is found that the curve for the Donnan correction is almost identical with the curve for the observed osmotic pressure. In other words, it turns out that the increase in osmotic pressure of a 1 per cent solution of originally isoelectric gelatin upon the addition of little acid until a maximum is reached, and the diminution of osmotic pressure upon the addition of further acid, are not due to any variation in the state of dispersion of the protein, but purely to the fact that protein ions cannot diffuse through the collodion membrane which is easily permeable to crystalloidal ions, as a consequence of which the molar concentration of the crystalloidal ions must always be greater inside the protein solution than outside. What varies with the pH of the gelatin solution is the quantity of the excess of $2y + z$ over $2x$. This follows from the Donnan equation (1), according to which

$$x = \sqrt{y^2 + yz} \text{ or } 2x = \sqrt{4y^2 + 4yz},$$

while

$$2y + z = \sqrt{4y^2 + 4yz + z^2}.$$

Now it is obvious that

$$\sqrt{4y^2 + 4yz + z^2} > \sqrt{4y^2 + 4yz},$$

i.e., the concentration of the crystalloidal ions inside the protein solution $2y + z$ is always greater than the concentration of the crystalloidal ions outside.

If we substitute for the term $2y + z - 2x$ of the Donnan correction the identical term

$$\sqrt{4y^2 + 4yz + z^2} - \sqrt{4y^2 + 4yz},$$

we can visualize why the osmotic pressure is a minimum at the isoelectric point, why it increases with the addition of little acid, reaching a maximum, and why it diminishes again with the addition of more acid.

At the isoelectric point no protein is ionized and, z being zero, the whole term

$$\sqrt{4y^2 + 4yz + z^2} - \sqrt{4y^2 + 4yz}$$

becomes zero. Hence, at the isoelectric point the observed osmotic pressure is purely that due to the protein, which is very low on account of the high molecular weight of gelatin.

When little acid, *e.g.*, HCl, is added to the solution of isoelectric gelatin, gelatin chloride is formed and some free acid remains, due to hydrolytic dissociation. Hence, both z (the concentration of Cl ions in combination with protein) and y (the concentration of Cl ions of the free HCl existing through hydrolysis) increase, but z increases at first more rapidly than y , and hence the excess of concentration of ions inside over that of ions outside increases until the greater part of protein is transformed into protein chloride, when the excess of crystalloidal ions inside over those outside reaches a maximum. From then on, z increases comparatively less than y with further addition of acid, so that z becomes finally negligible in comparison with y . This explains why the Donnan correction becomes zero again when enough acid is added, and why the observed osmotic pressure becomes as low again as at the isoelectric point.

We will now show that these mathematical deductions from Donnan's equation agree quantitatively with the facts. For this purpose it is necessary to calculate the Donnan correction $2y + z - 2x$, *i.e.*, the osmotic pressure due to the excess of molar concentration of crystalloidal ions (H and Cl) inside the gelatin chloride solution over the molar concentration of H and Cl ions outside. As stated, y and x can be calculated from the measurements of pH inside and pH outside respectively and z can be calculated from x and y according to equation (1),

$$z = \frac{(x + y)(x - y)}{y}.$$

The theoretical osmotic pressure of a gram-molecular solution is in terms of millimeter pressure of a column of water (corrected for 24°C.),

$$22.4 \times 760 \times 13.6 \times \frac{293}{273} = 2.5 \times 10^5 \text{ mm.}$$

In other words, a theoretical pressure of 2.5 mm. H₂O corresponds to a concentration of 10^{-5} N and in order to get the Donnan correction we have to multiply $2y + z - 2x$ by 2.5×10^{-5} .

Table XXVI gives the results of an experiment with gelatin chloride. The values of pH inside are the pH of the gelatin

solutions at the end of the experiment and the values of pH outside are the pH of the outside aqueous solutions (free from gelatin) at the end of the experiment (after 18 hours). The next rows give the values of y , x , and z , the sixth row gives the values of $2y + z - 2x$, and the next row the Donnan correction for the

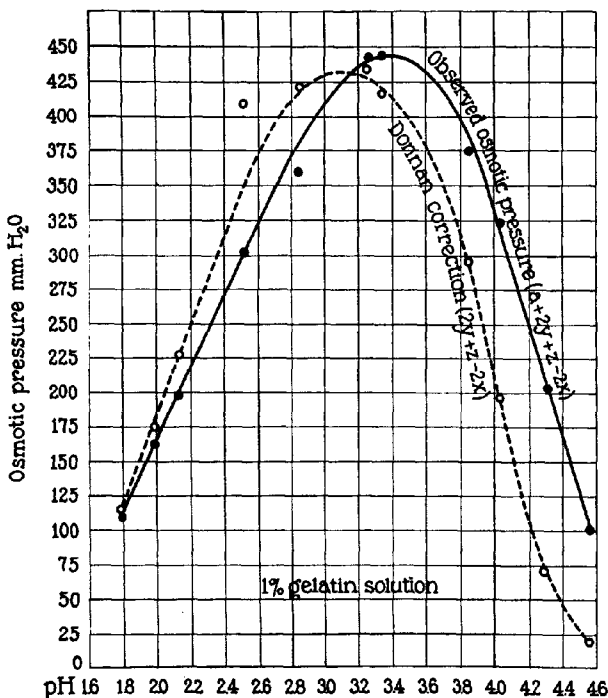


FIG. 67.—Showing agreement and minor discrepancies between the curves of observed and calculated osmotic pressures of 1 per cent gelatin chloride solutions.

osmotic pressure of the solution, namely, $2.5 \times 10^5 (2y + z - 2x)$. The last row gives the observed osmotic pressure. The point of interest is the comparison of the Donnan correction with the observed osmotic pressure. The graph in Fig. 67 is intended to facilitate this comparison. The abscissæ are the pH of the gela-

TABLE XXVI.—ONE PER CENT GELATIN CHLORIDE
(Comparison between observed osmotic pressures and Donnan correction)

pH inside.....	4.56	4.31	4.03	3.85	3.33	3.23	2.85	2.52	2.13	1.99	1.79	1.57
pH outside.....	4.14	3.78	3.44	3.26	2.87	2.81	2.53	2.28	2.00	1.89	1.72	1.53
$v = C_B \text{ inside} \times 10^6$	2.7	4.9	9.3	14.1	46.8	56.2	141.0	302.0	741.0	1,023.0	1,622.0	2,682.0
$z = C_B \text{ outside} \times 10^6$	7.2	16.6	36.3	54.9	135.0	155.0	265.0	524.0	1,000.0	1,288.0	1,905.0	2,951.0
$z = \frac{(x+v)(x-v)}{y}$	10.5	51.4	132.5	200.0	343.0	372.0	477.0	608.0	609.0	600.0	612.0	544.0
$2y + z - 2z$	7.5	28.0	78.5	118.4	166.6	174.4	169.0	164.0	91.0	70.0	46.0	26.0
Donnan correction.....	19.0	70.0	196.0	296.0	416.0	436.0	423.0	410.0	227.0	175.0	115.0	65.0
Observed osmotic pressure.....	100.0	202.0	322.0	373.0	443.0	442.0	360.0	308.0	198.0	162.0	110.0	90.0

TABLE XXVII.—ONE PER CENT GELATIN PHOSPHATE
(Comparison between observed osmotic pressures and Donnan correction)

pH inside.....	4.79	4.54	4.31	3.98	3.68	3.56	3.38	3.24	3.02	2.67	2.42	1.92	1.74
pH outside.....	4.70	4.10	3.77	3.40	3.14	3.04	2.90	2.80	2.66	2.39	2.22	1.98	1.67
$v = C_B \text{ inside} \times 10^6$	1.6	2.9	4.9	10.5	20.9	27.5	41.7	57.5	95.5	213.8	380.2	758.6	1,820.0
$z = C_B \text{ outside} \times 10^6$	2.0	7.9	16.9	39.8	72.4	91.2	125.9	158.5	218.8	407.4	602.6	1,047.0	2,138.0
$z = \frac{(x+v)(x-v)}{y}$	0.9	18.6	53.3	140.0	228.0	231.0	338.0	380.0	405.0	556.0	575.0	686.0	690.0
$2y + z - 2z$	0.1	8.6	31.3	81.4	125.0	103.6	169.6	178.0	158.0	169.0	130.0	109.0	54.0
Donnan correction.....	22.0	77.0	203.0	310.0	253.0	423.0	445.0	395.0	420.0	374.0	273.0	157.0	135.0
Observed osmotic pressure.....	34.0	111.0	199.0	328.0	416.0	420.0	426.0	436.0	401.0	350.0	275.0	190.0	121.0

tin solution at equilibrium taken from the first row in Table XXVI. The ordinates of one curve are the observed osmotic pressures ($a + 2y + z - 2x$), and of the second curve the osmotic pressures calculated from the Donnan correction ($2y + z - 2x$). These two curves are, with the exception of certain discrepancies to be discussed later, almost identical. Both are a minimum at the isoelectric point, both rise to almost the same height of about 440 mm., and both drop again to the low level from which they started at the isoelectric point. There are two discrepancies, first, the ascending branch of the observed pressure (from pH 4.7 to pH 3.4) is higher by an almost constant value than the ascending branch of the curve for the Donnan correction. This constant difference is probably the value of a , *i.e.*, the osmotic pressure of the 1 per cent gelatin solution, and, if this surmise is correct, it means that the osmotic pressure of the protein in solution is not changed by the variation in pH. The rise and fall of the curve of observed osmotic pressure are solely caused by the variation in the excess of the concentration of H and Cl ions inside the protein solution over that outside, *i.e.*, by the variation of the value $2y + z - 2x$ with the pH. If it were not for this effect the osmotic pressure of the protein solution would be nearly constant, or would vary within narrow limits. Second, the differences of the descending branch of the two curves in Fig. 67 (from pH 3.4 to pH 1.8) are probably due to errors in the calculation of z . We shall see later that a slight error in pH inside or outside causes a considerable error in the value of z , when the pH is comparatively low.

The acid either does not affect the osmotic pressure of the protein itself or does so only to a small extent. What appeared as a colloidal effect of the acid on the protein is, therefore, only the effect of the Donnan equilibrium on the distribution of crystallizable diffusible ions on opposite sides of the membrane.

Table XXVII gives the osmotic pressures and the Donnan correction if the acid used is H_3PO_4 , and it is obvious that both sets of values are again in close agreement. Figure 68 gives the graphs, the abscissæ being the pH of the gelatin phosphate solution at equilibrium, and the ordinates of one curve are the observed osmotic pressures of the gelatin phosphate solution, while the ordinates of the second curve are the values of the Donnan correc-

tion for the gelatin phosphate solution, according to the values in Table XXVII. The reader will notice that the curve for the observed osmotic pressures and that for the osmotic pressures calculated from the Donnan correction are again similar and almost identical, except for the slight differences already dis-

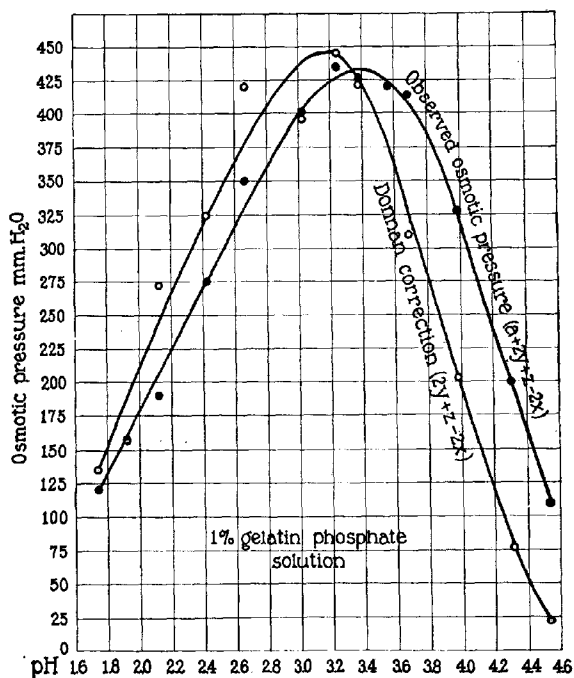


FIG. 68.—Showing agreement and minor discrepancies between the curves of observed and calculated osmotic pressures of 1 per cent gelatin phosphate solutions.

cussed in connection with the gelatin chloride curves. As a matter of fact the curve for the observed osmotic pressure for gelatin phosphate is identical with the curve for the observed osmotic pressure for gelatin chloride; and the curves for the osmotic pressures of the Donnan correction in the case of gelatin

chloride and gelatin phosphate are also identical. This latter fact is of great importance, because it is the proof of the valency rule, which we found to replace the Hofmeister series in regard to the ion effect on the colloidal behavior of protein solutions. If this latter effect is, as we maintain, purely the consequence of the unequal distribution of crystalloidal ions on the opposite sides of the membrane as demanded by the Donnan equation, it must be the same in the case of all acids with monovalent anion, since only the valency, and not the nature of the anion, enters into the Donnan equation for protein-acid salts. The mistake the colloid chemists have made is that they have assumed that the anion of the acid acts on the colloidal properties of the protein, which is not true. Since the anion in the case of gelatin phosphate is the monovalent anion H_2PO_4 , on the basis of the Donnan theory the influence of H_3PO_4 on the osmotic pressure of gelatin must be identical with the influence of HCl on the osmotic pressure, which is shown to be true by a comparison of Figs. 67 and 68.

THE VALENCY EFFECT

We now come to the valency effect. The curve for the osmotic pressure of a gelatin sulphate solution is only about half as high at the maximum as the curve for gelatin chloride, because the anion of gelatin sulphate is bivalent.

The term $2y + z - 2x$ for the Donnan correction holds for solutions of all gelatin-acid salts with monovalent anion, *i.e.*, gelatin chloride, acetate, phosphate, tartrate, citrate, etc. When, however, the anion of a gelatin-acid salt is divalent, as in the case of gelatin sulphate, the equilibrium equation becomes one of the third degree, as has been stated in the preceding chapter. If x is the molar hydrogen ion concentration of the outside solution, the molar concentration of the SO_4 ions in the outside solution becomes $\frac{x}{2}$. If y is the molar concentration of the H ions of the free H_2SO_4 in the inside solution, $\frac{y}{2}$ is the concentration of the SO_4 ions of the free acid inside the gelatin sulphate solution. In the case of gelatin chloride z represented the molar concentration of chloride ions in combination with the gelatin; hence $\frac{z}{2}$ will

represent the molar concentration of SO_4 ions in combination with the same number of gelatin ions.

The equilibrium equation, therefore, assumes in the case of gelatin sulphate the following form:

$$x^2 \cdot \frac{x}{2} = y^2 \frac{(y+z)}{2}. \quad (2)$$

From equation (2) it follows that

$$z = \frac{x^3 - y^3}{y^2}.$$

The excess of molar concentration of crystalloidal ions inside over that outside is, therefore, in the case of gelatin sulphate

$$\frac{3}{2}y + \frac{z}{2} - \frac{3}{2}x.$$

From this term the Donnan correction was calculated for the gelatin sulphate, as shown in Table XXVIII. The comparison

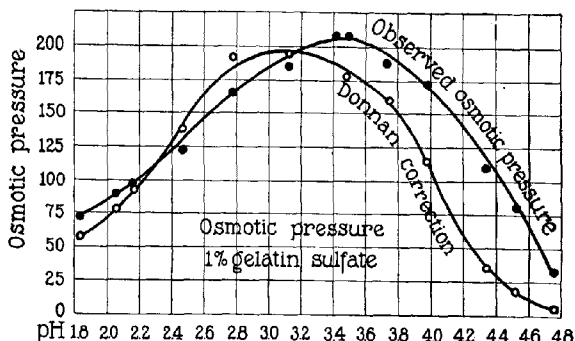


FIG. 69.—Showing agreement and minor discrepancies between the curves of observed and calculated osmotic pressures of 1 per cent gelatin sulphate solutions.

between observed osmotic pressure and the osmotic pressure calculated from the Donnan correction is shown graphically in Fig. 69. It is obvious that the osmotic pressure calculated from the Donnan correction is practically identical with the observed osmotic pressure, except for the constant excess of the ascending branch of the curve for observed osmotic pressure over that of the Donnan correction. The maximum osmotic pressure of a gelatin sulphate solution is only 200 mm. *i.e.*, a little less than

half of the observed osmotic pressure of either gelatin chloride or gelatin phosphate solution (of the same concentration of originally isoelectric gelatin). The same is true for the Donnan correction for gelatin sulphate. The agreement of the Donnan correction with the observed osmotic pressure leaves no doubt that the greater depressing action of SO_4 —which in the colloidal literature is generally referred to as the specific “dehydrating” effect of SO_4 —is simply the consequence of the fact that the equilibrium equation for gelatin sulphate is an equation of the third degree, while the equilibrium equation for gelatin chloride or gelatin phosphate is a quadratic equation. At the same pH and the same concentration of originally isoelectric gelatin, the value for the Donnan correction for gelatin chloride or gelatin phosphate is about twice that of the Donnan correction for gelatin sulphate.

It may be briefly stated that the effects of seven weak monobasic acids (HCl, HBr, HI, HNO_3 , lactic, acetic, and propionic acids) on the osmotic pressure of gelatin solutions containing 1 gm. dry weight of originally isoelectric gelatin were found to be identical when plotted over the pH of the gelatin solutions; the influence of the two strong dibasic acids, H_2SO_4 and sulphosalicylic acid, on the osmotic pressure was also found to be identical, but about half as high as the values for the influence of the monobasic acids on the osmotic pressure of the gelatin solutions at the same pH of the gelatin solutions.

THE CALCULATION OF pH OUTSIDE FROM THE OBSERVED OSMOTIC PRESSURE

The osmotic pressure P of the Donnan correction was in the case of gelatin chloride $P = 2.5 \times 10^5(2y + z - 2x)\text{mm.}$ water. Dr. Hitchcock has called the writer's attention to the fact that we can use the observed values of the osmotic pressure to calculate the pH inside or outside with the aid of this expression and compare it with the directly observed pH. If our theory is correct the observed and calculated pH should agree very closely.

If we make temporarily the assumption that the observed osmotic pressure P of a gelatin chloride solution is identical with the Donnan correction, then

$$z = \frac{P}{2.5 \times 10^5} - 2y + 2x.$$

If we substitute this value in the Donnan equation (1), we get

$$x^2 = y \left(y + \frac{P}{2.5 \times 10^5} - 2y + 2x \right).$$

The correctness of the temporary assumption that the observed osmotic pressure is practically identical with the Donnan correction can be tested by using the observed value either for x or for y and solving for the other. Solving for x we get

$$x^2 = y \left(y + \frac{P}{250,000} - 2y + 2x \right) = -y^2 + 2yx + \frac{Py}{250,000}$$

$(x - y)^2 = \frac{Py}{250,000}$ where x and y are expressed in mols per liter as usual.

$$x = y + \frac{\sqrt{Py}}{500}. \quad (2)$$

Using the values for y obtained from the measurement of pH inside and using the observed values for the osmotic pressure for P , it is possible to calculate the values for x from equation (2). These values when translated into terms of pH (pH outside = $-\log x$) can be compared with the observed values for the pH outside.

Table XXIX compares the values for pH outside calculated from the data for P and for pH inside given in Table XXVI with the observed values for pH outside.

TABLE XXIX.—ONE PER CENT GELATIN CHLORIDE
(pH outside calculated from equation $x = y + \frac{Py}{500}$)

Observed pH outside.....	4.14	3.78	3.44	3.26	2.87	2.81	2.53	2.28	2.00	1.89	1.72	1.53
Calculated pH outside.....	3.88	3.61	3.36	3.22	2.86	2.81	2.55	2.31	2.01	1.89	1.72	1.52

It is obvious that the observed and calculated values for pH outside are identical for all pH below 3.2 and that even at an observed pH 3.26 the difference is small. It is larger when the pH outside is above 3.4, *i.e.*, before the maximum osmotic pressure is reached. This corresponds to the ascending branch of the curve in Fig. 67.

The pH outside was also calculated for gelatin phosphate solutions from the values of P and pH inside given in Table XXVII. Table XXX gives the comparison of these calculated

values with the observed values for pH outside. It is again obvious that the calculated and observed values agree very closely below pH 3.14, while they do not agree when the pH is above 3.14.

If we now compare these results with the curves in Figs. 67 and 68, it is obvious that the discrepancies between the descending branches of the Donnan correction and the observed osmotic pressures were due to errors in calculating z ; while the discrepancy in the ascending branch had a different reason, namely, that this discrepancy is partly or entirely the expression of the true osmotic pressure of the protein solution, which does not enter into the Donnan correction, but which, of course, is a fraction of the observed osmotic pressure.

TABLE XXX.—ONE PER CENT GELATIN PHOSPHATE

(pH outside calculated from equation $x = y + \frac{\sqrt{Py}}{500}$)

Observed pH outside.....	4.70	4.10	3.77	3.40	3.14	3.04	2.90	2.80	2.66	2.39	2.22	1.98	1.83	1.67
Calculated pH outside.....	4.20	3.85	3.61	3.32	3.10	3.02	2.90	2.80	2.66	2.41	2.23	2.00	1.83	1.67

This agreement leaves little doubt that the explanation of the influence of acid on the osmotic pressure of protein solutions on the basis of the Donnan equilibrium is correct.

THE OSMOTIC PRESSURE OF SOLUTIONS OF CASEIN CHLORIDE

Similar experiments were made with casein chloride. The material used was casein prepared from skimmed milk after the method of Van Slyke and Baker.¹ The finely powdered casein used by us was nearly isoelectric. Casein is only sparingly soluble in water at the isoelectric point, but it becomes more soluble in hydrochloric or phosphoric acid if enough of the acid is added. Portions of 1 gm. of powdered casein were put into 100 c.c. of water containing various quantities of 0.1 N HCl (see first row in Table XXXI). After 24 hours all the casein was dissolved in those solutions which contained more than 5 and less than 40 c.c. of 0.1 N HCl in 100 c.c. The hydrogen ion concentration of the casein chloride solutions was determined after the casein was dis-

¹ VAN SLYKE, L. L. and BAKER, J. C., *J. Biol. Chem.*, vol. 35, p. 127, 1918.

TABLE XXXI.—DIFFERENCE OF HYDROGEN ION CONCENTRATION BETWEEN CASEIN SOLUTION AND OUTSIDE SOLUTION AT EQUILIBRIUM

at equilibrium																																														
1. C.c. of 0.1 N HCl contained in 100 c.c. of solution.....																																														
2. Appearance of solution.....																																														
Precipitate					Slight precipitate		Solution										Precipitate																													
1	2	3	4	5	6	7	8	10	12.5	15	17.5	20	25	30	40																															
pH at beginning of experiment																																														
3. pH of casein (inside) solution.....					3.10												2.94		2.78		2.64		2.45		2.26		2.13		2.02		1.93		1.81		1.71											
4. pH of outside solution.....					3.20												3.00												2.90		2.60		2.30		2.20		2.10		1.95		1.80		1.70			
pH after 18 hours (at equilibrium)																																														
5. pH of casein (inside) solution.....					3.46												3.32												3.22		2.93		2.78		2.62		2.52		2.39		2.22		2.04		1.89	
6. pH of outside solution.....					3.13												2.97												2.88		2.67		2.57		2.42		2.35		2.26		2.14		2.00		1.88	

TABLE XXXII.—APPROXIMATE AGREEMENT BETWEEN OBSERVED OSMOTIC PRESSURE AND OSMOTIC PRESSURE CALCULATED FROM DONNAN'S EQUATION

1. pH of casein solution at equilibrium.....	1.73	1.89	2.04	2.22	2.39	2.52	2.62	2.78	2.93	3.22	3.32	3.46	3.61	3.68	3.87	4.04
2. $Ca^{+} \times 10^4$ inside (<i>y</i>).....	1.862	1.283	912	603	407	302	240	186	118	60.3	47.9	34.7	24.6	20.9	13.5	9.1
3. $Ca^{+} \times 10^4$ outside (<i>x</i>).....	1.862	1.318	1,000	724	550	447	380	269	214	132	107	74.1	52.5	38.0	20.4	14.5
4. $(x + y)(x - y) = z$	0	61	185	267	337	360	362	270	270	229	191	124	87.5	48.1	17.3	14.0
5. $2y + z - 2x$	0	1	9	25	51	70	82	64	78	86	73	45	32	14	3.5	3.2
6. Donnan correction.....	0	2.5	22.5	62.5	128	175	205	160	165	215	183	113	80	35	8.7	9
7. Observed osmotic pressure, mm. of solution...	41	69	85	102	126	145	153	177	187	189	173	135	77	43	23	14

solved and each collodion bag containing a casein chloride solution was dipped into a beaker containing 350 c.c. of HCl of originally the same hydrogen ion concentration as that of the casein solution.

The first row in Table XXXI gives the volume of 0.1 N HCl originally contained in 100 c.c. of solution with 1 gm. of originally approximately isoelectric casein. The next row states whether or not all the casein went into solution in the next 24 hours, that is, whether or not there was a precipitate at the bottom of the beaker containing the solution. It is evident that no more precipitate was left when the solution contained more than 5 and less than 40 c.c. of 0.1 N HCl in 100 c.c. The third row gives the pH of the casein solutions at the beginning of the osmotic pressure experiment. The next row gives the pH of the outside solution at the beginning. It was approximately identical in each case with that of the inside solution.

The important part of the table is the last two rows (5 and 6), giving the pH values of the inside and outside solutions *after osmotic equilibrium was established*, that is, after 18 hours. The pH has changed both in the inside and outside solutions, but it is without exception smaller in the outside than in the inside solution; or, in other words, the outside solution has at equilibrium a higher hydrogen ion concentration than the inside solution. This is exactly what should happen according to Donnan's formula for membrane equilibria as stated above.

In row 1, Table XXXII, are given the observed values of the pH of the casein solutions as found at the end of the experiment after equilibrium was established. It is obvious that the osmotic pressure is a minimum nearest the isoelectric point (pH 4.04), that it rises with the increase of acid until the maximal osmotic pressure is reached at about pH 3.0, and that the osmotic pressure falls again when the hydrogen ion concentration in the solution increases beyond this point. Row 6 gives the Donnan correction, and row 7 the observed osmotic pressure.

A comparison of the corresponding values of these two latter rows of Table XXXII shows that, with the limits of accuracy of the experiments and calculations, there is little difference between the Donnan correction and the observed osmotic pressure, or, in other words, the influence of acid on the osmotic pressure of

casein solutions is entirely due to the Donnan equilibrium. That share of the osmotic pressure which in this experiment was due to the casein molecules, ions, or aggregates was so small that it was within the limit of error of measurement and calculation.

In comparing the observed and calculated values for osmotic pressure in Table XXXII the discrepancies appear to be rather large. This is again simply due to the fact that a slight variation of the pH measurements in the second decimal (so slight that it is inside the limit of the source of error) causes a considerable variation in the calculated value for the Donnan correction, $2y + z - 2x$.

If we assume that the observed osmotic pressure is identical with the Donnan correction, we can calculate the pH of the outside solution from the observed osmotic pressure and the measurement of pH inside from the formula given above,

$$x = y + \frac{\sqrt{Py}}{500}.$$

These values are given in Table XXXIII and the reader will notice that the agreement between observed and calculated pH outside is perfect between pH of the outside solution of 3.42 and 2.14, and fair down to pH 1.73. The values between 4.7 (the isoelectric point of casein) and pH 3.42 are the region where less than 1 gm. of casein goes into solution in HCl.

TABLE XXXIII.—COMPARISON OF OBSERVED VALUES FOR pH OUTSIDE WITH THOSE CALCULATED ON THE ASSUMPTION THAT THE OSMOTIC PRESSURE OBSERVED WAS DUE ENTIRELY TO THE DONNAN EQUILIBRIUM

Observed pH.....	1.73	1.88	2.00	2.14	2.26	2.35	2.42	2.57
Calculated pH.....	1.69	1.83	1.96	2.12	2.26	2.36	2.44	2.56
Observed pH.....	2.67	2.88	2.97	3.13	3.28	3.42	3.69	3.84
Calculated pH.....	2.67	2.84	2.98	3.11	3.28	3.40	3.61	3.79

These results leave no doubt that the influence of acid on the osmotic pressure of casein solutions is exclusively due to the Donnan equilibrium.

It is worthy of notice that the agreement is not good for pH above 3.40, but becomes perfect below this value. This agrees with the observations on gelatin solutions.

THE INFLUENCE OF THE ADDITION OF SALTS

It was first pointed out by R. S. Lillie¹ that the addition of salt to a gelatin solution depresses its osmotic pressure. It should, however, be stated that this depressing effect does not occur at

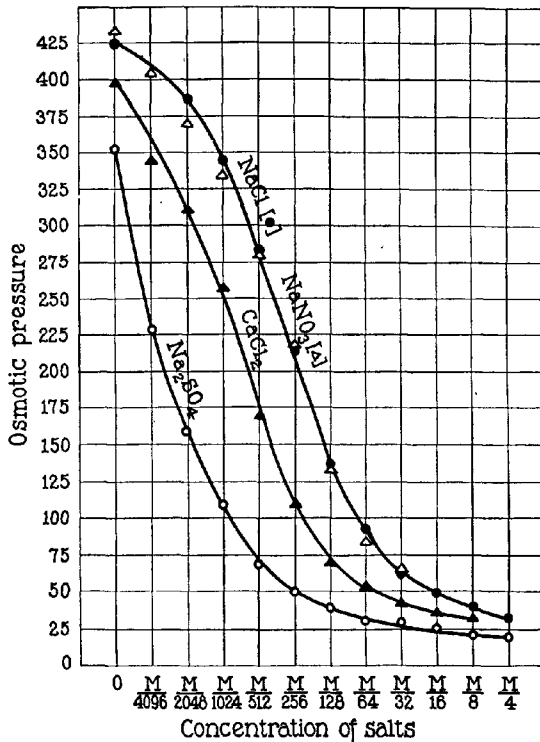


FIG. 70.—Depressing effect of neutral salts on the osmotic pressure of a 1 per cent solution of gelatin chloride of pH 3.5.

the isoelectric point. When we add different salts to a gelatin chloride solution of an initial pH 3.5 containing 1 gm. of originally isoelectric gelatin in a 100-c.c. solution, the depressing effect of the salt on osmotic pressure should according to the Donnan

¹ LILLIE, R. S., *Am. J. Physiol.*, vol. 20, p. 127, 1907-08.

equation be due to the anion; and this is the case, as Fig. 70 shows. The gelatin chloride solutions were made up in different concentrations of the salts, NaCl, NaNO₃, CaCl₂, and Na₂SO₄. The pH of the mixtures was always 3.5. Collodion bags of a volume of about 50 c.c. were filled with the gelatin chloride-salt mixtures. These bags were dipped into beakers containing 350 c.c. of a solution of the same inorganic salt of the same concentration as that contained in the gelatin solutions, but these outside solutions contained no gelatin. The pH of the outside solutions was made at the beginning 3.0 to accelerate the establishment of the equilibrium. The osmotic pressure was read after about 20 hours. The temperature was (as always in these cases) 24°C.

In Fig. 70 the abscissæ are the initial concentrations of the salt solutions, while the ordinates are the osmotic pressures. The Donnan equilibrium caused a change of pH as well as of the distribution of the neutral salts on the opposite sides of the membrane. The change of pH in this experiment has already been discussed in Tables XIX, XX and XXI of Chap. XI. Figure 70 shows that the depressing effect of NaCl and NaNO₃ is practically the same, that the depressing effect of an equimolecular concentration of CaCl₂ is about twice as great as that of NaCl, but that the effect of Na₂SO₄—where the anion is bivalent—is about eight times as great as that of a NaCl solution of the same molecular concentration. This leaves no doubt that the depressing effect is due to the anion and that the cation is seemingly without any influence (it has certainly not any influence in the opposite direction from that of the anion).

Further experiments have shown that the influence of NaCl, CaCl₂, and LaCl₃ on the osmotic pressure of gelatin chloride solutions of a given pH is exactly the same for equal concentrations of Cl ions of these salts.

According to the writer's theory, this depressing effect of salt on the osmotic pressure of a solution of a protein salt should be entirely due to the influence of the salt on the value of the Donnan correction which is, in the case of gelatin chloride,

$$2y + z - 2x,$$

for which we can substitute

$$\sqrt{4y^2 + 4yz + z^2} - \sqrt{4y^2 + 4yz}.$$

When a salt like NaCl is added to a solution of gelatin chloride, the value of z is certainly not increased, but the value of y is increased, if by y we understand the concentration of all the free Cl ions of the salt and the acid; and the increase of y is the greater the more salt is added. Hence with addition of salt z becomes increasingly negligible in comparison with y , and hence the term

$$\sqrt{4y^2 + 4yz + z^2} - \sqrt{4y^2 + 4yz}$$

must become increasingly smaller, finally approaching zero. In other words, the depressing action of the addition of salt on the osmotic pressure of protein solutions is due not to a depression of the osmotic pressure of the protein but to a diminution of the excess of the concentration of crystalloidal ions inside the protein solution over that outside.

THE INFLUENCE OF THE CONCENTRATION OF A PROTEIN SOLUTION UPON THE OSMOTIC PRESSURE

An increase in the concentration of a protein solution at the same pH and in the absence of neutral salts should have a double effect on the osmotic pressure. It should first, raise the osmotic pressure of the solution on account of the increase in the number of protein particles in the solution; and it should second, lead to a further increase in osmotic pressure due to an increase in the value of $2y + z - 2x$ or $\sqrt{4y^2 + 4yz + z^2} - \sqrt{4y^2 + 4yz}$, for it is obvious that as long as y is constant, *i.e.*, at constant pH of the gelatin solution, the value of the term $\sqrt{4y^2 + 4yz + z^2} - \sqrt{4y^2 + 4yz}$ will increase with increasing z . The two effects can be separated by subtracting the value of the term $2y + z - 2x$ from the observed osmotic pressure. The difference between the two values should (within the limits of the accuracy of the experiments) increase with the concentration of the protein. Both expectations are fulfilled.

Different concentrations of gelatin phosphate from 2 to 0.5 per cent were prepared, all having a pH of 3.5. The gelatin phosphate solutions were put into collodion flasks of 50-c.c. volume, each connected with a glass tube serving as a manometer as described, and these flasks were put into beakers containing 350 c.c. of H_2O , the pH of which was brought at the beginning of the experiment to 3.5 through the addition of H_3PO_4 . When the

TABLE XXXIV.—INFLUENCE OF CONCENTRATION OF GELATIN PHOSPHATE OF PH OF ABOUT 3.6 ON THE OSMOTIC PRESSURE
(All experiments were made in duplicate)

	Concentration of gelatin in per cent									
	2	2	1½	1½	1	1	¾	¾	½	½
pH inside at equilibrium.....	3.64	3.66	3.60	3.60	3.65	3.66	3.60	3.60	3.61	3.62
pH outside at equilibrium.....	3.02	3.02	3.02	3.01	3.12	3.11	3.14	3.12	3.21	3.19
$y = C_H \text{ inside} \times 10^5$	22.9	21.9	25.1	25.1	22.4	21.9	25.1	25.1	24.6	24.0
$x = C_H \text{ outside} \times 10^5$	95.5	95.5	95.5	97.7	75.9	77.6	72.4	75.9	61.7	64.6
$z = \frac{y}{(x+y)(x-y)}$	375.0	395.0	338.0	355.0	235.0	253.0	184.0	204.0	130.0	150.0
$2y + z - 2x$	230.0	248.0	197.0	210.0	128.0	142.0	89.0	102.0	56.0	69.0
Observed osmotic pressure.....	860.0	860.0	715.0	680.0	420.0	445.0	314.0	316.0	186.0	186.0
Donnan correction.....	576.0	620.0	493.0	523.0	320.0	355.0	222.0	255.0	140.0	172.0
Difference (osmotic pressure due to gelatin)...	284.0	240.0	222.0	157.0	100.0	90.0	92.0	61.0	46.0	14.0
Mean.....	262.0		190.0		95.0		73.0		26.0	

bags containing gelatin phosphate solutions are put into water, the latter diffuses rapidly into the gelatin solution, thereby lowering the concentration of the gelatin solution. To avoid this error so much gelatin phosphate solution was poured into each bag and glass tube that at the beginning of the experiment the liquid reached already to about that level which from preceding experiments we knew the gelatin solution would reach in the manometer at the point of osmotic equilibrium. All experiments were made in duplicate. In addition to the osmotic pressure we measured the pH inside and outside after equilibrium was reached. From these latter data the Donnan correction could be calculated, being equal to

$$(2y + z - 2x) \times 2.5 \text{ mm. H}_2\text{O}.$$

By deducting this value from the observed osmotic pressure in each case it was hoped to obtain a rational value for the share of the protein particles in the observed osmotic pressure. Table XXXIV gives the results.

First, it may be pointed out that the Donnan correction increases with the concentration of the protein as the theory demands.

The reader's attention is called to the last two rows of figures (Table XXXIV) giving the difference between the observed osmotic pressures and the Donnan correction, since if this difference actually represents the osmotic pressure due to the gelatin particles, the figures should be in direct proportion to the concentration of the gelatin. The experiments were all made in duplicate to give some idea of the magnitude of error, and it is obvious that the error may be considerable, 25 per cent or more, because the errors in the observed and the calculated values are additive. Thus the "difference" is for 0.75 per cent solution in one case 92, in the other 61, a variation of 50 per cent! If we take this into consideration, we may conclude that the differences between the observed and the calculated osmotic pressures are compatible with the idea that the difference is the value for the osmotic pressure due to the gelatin particles in solution.

A similar experiment was made with different concentrations of solutions of the chloride of crystalline egg albumin. The original pH of the albumin chloride solution was 3.5 and that of the outside

TABLE XXXV.—INFLUENCE OF CONCENTRATION OF ALBUMIN CHLORIDE OF pH OF ABOUT 3.4 ON THE OSMOTIC PRESSURE

	Concentration of egg albumin in per cent					
	4	3	2	1	$\frac{1}{2}$	$\frac{1}{4}$
pH inside at equilibrium.....	3.34	3.32	3.38	3.40	3.40	3.40
pH outside at equilibrium.....	2.98	2.97	3.07	3.14	3.19	3.24
$y = \text{Cl inside} \times 10^4$	45.7	47.9	41.7	39.8	39.8	39.8
$x = \text{Cl outside} \times 10^4$	104.7	107.2	85.1	72.4	64.5	57.5
$z = \frac{(x+y)(x-y)}{y}$	194.0	192.0	132.0	92.0	64.6	43.3
$2y + z - 2x$	76.0	74.0	45.0	27.0	15.0	8.0
Observed osmotic pressure.....	776.0	555.0 +	375.0	163.0	75.0	36.0
Donnan correction.....	190.0	185.0	113.0	67.0	39.0	20.0
Difference (osmotic pressure due to albumin)...	586.0	370.0 +	262.0	96.0	36.0	16.0

solution 3.0. After equilibrium was established, the pH both inside and outside was slightly changed, as is shown in Table XXXV. The osmotic pressures for 0.25 to 4 per cent solutions of albumin chloride were measured and calculated for $2y + z - 2x$. The difference, which should be the osmotic pressure of the albumin particles in solution, is found in the last row. It is almost identical with the difference found for gelatin chloride for the same concentration of gelatin.

DIRECT DETERMINATION OF THE VALUE FOR z

In the preceding calculations z was found with the aid of the Donnan equation

$$z = \frac{(x+y)(x-y)}{y}$$

where x and y were obtained from pH measurements. There exists a way of measuring z , namely, by determining the concentration of Cl inside a 1 per cent gelatin chloride solution by titration. The Cl inside is partly in combination with H (free HCl) and partly combined with gelatin. By titrating with NaOH to pH 7.0 and making the correction for isoelectric gelatin we determine the value $z + y$. y is known from the pH, and by

deducting y we get z . We made such determinations at the end of an osmotic experiment and calculated z also from

$$\frac{(x+y)(x-y)}{y}$$

in the same experiment. Table XXXVI gives a comparison of the values of z obtained in identical solutions by the two different methods.

TABLE XXXVI.—CONCENTRATIONS OF $z \times 10^5$ N

pH of gelatin solution.....	4.51	4.26	3.96	3.61	3.53	3.32	3.23	2.86	2.32	2.16	1.93
z calculated from $\frac{(x+y)(x-y)}{y}$	30	90	166	223	252	316	387	493	570	687	687
z found by titration.....	17	84.5	170	275	291	342	401	532	548	838	885

This table shows that the values for z calculated by way of the Donnan equilibrium are in good agreement with the values for z found by titration, except when the pH becomes too low. This latter fact was to be expected, since a difference of one or two units in the second decimal of the pH measurement causes a greater variation of the concentration (calculated from the pH) the lower the pH.

Summarizing the results of this chapter, we may state that the influence of electrolytes on the osmotic pressure of solutions of protein salts—gelatin, crystalline egg albumin, casein, and, according to Dr. Hitchcock, also edestin—is due to the fact that on account of the inability of protein ions to diffuse through membranes which are permeable to crystalloidal ions the total molar concentration of the crystalloidal ions is always greater inside the protein solution than outside, and that this difference varies with pH, valency of ions, and concentration of salts. This difference can be determined with the aid of Donnan's equation for membrane equilibria and it is shown that the osmotic pressure calculated from the excess of the concentration of crystalloidal ions inside the protein solution over that outside represents practically the total influence of electrolytes on the osmotic pressure of protein solutions.

CHAPTER XIII

SWELLING

I. THE MEMBRANE POTENTIALS OF SOLID JELLIES OF GELATIN

1. General Remarks

Procter and Wilson¹ have developed an osmotic theory of swelling of gelatin on the basis of Donnan's theory of membrane equilibria, which preceded the writer's work on the same subject. Their theory consists briefly in this, that when HCl is added to gelatin, gelatin chloride is formed, which dissociates electrolytically, and that the gelatin ion cannot diffuse out from the jelly, while the jelly is easily permeable to the ions of the acid. As a consequence, a membrane equilibrium is set up between the liquid inside the jelly and the outside solution, whereby the concentration of crystalloidal ions is greater inside the jelly than in the aqueous solution surrounding the jelly. This excess of the osmotic pressure of the solutions of crystalloids inside the jelly over that outside leads to a diffusion of water into the gel, whereby the latter swells. The limiting force for the swelling is the force of cohesion between the molecules of the jelly of gelatin.

They were able to prove this theory quantitatively and their work was the first application of Donnan's equilibrium theory to the explanation of colloidal phenomena. Their proof is so convincing that it is difficult to understand why their osmotic theory of swelling was not at once universally accepted. There were, perhaps, two reasons, the first one being that many chemists were reluctant to believe that proteins form true ionizable salts with acids and alkalies. This difficulty no longer exists. The second difficulty was that the application of Donnan's theory of membrane equilibria demanded the proof that there exists a definite membrane potential between the jelly and the surrounding aqueous solution, and this proof was lacking. It will be our

¹ PROCTER, H. R., and WILSON, J. A., *J. Chem. Soc.*, vol. 109, p. 307, 1916.

first task to furnish the proof for the existence of these membrane potentials and for their quantitative connection with Donnan's equation.

The condition for the establishment of a membrane potential is the existence of a block to the diffusion of one type of ions, while no such block exists for other crystalloidal ions. When acid is added to solid isoelectric gelatin, the solid gelatin is ionized in the same way as if it were in solution. The block which prevents the diffusion of gelatin ions from the gel consists in the forces of cohesion between certain "oily" groups of the gelatin molecules

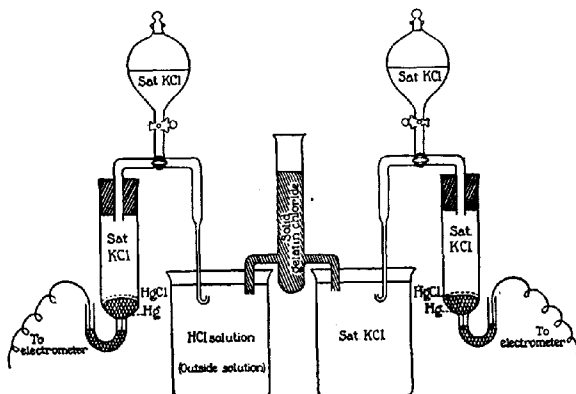


FIG. 71.—Method of measuring the P.D. between gel and surrounding solution.

or ions, which are the cause of the jelly formation. This block gives rise to the Donnan equilibrium which leads to the increase in osmotic pressure and to the establishment of a P.D. between the jelly and the solution surrounding it.

The method of our experiments was as follows: Powdered particles of isoelectric gelatin were put into a solution of acid or alkali at a temperature of 20°C. and allowed to remain there for a number of hours in order to bring about a complete or approximate equilibrium between the inside of the micellæ and the outside solution.¹ The temperature must not be above 20°C., since otherwise the granules of gelatin will dissolve too rapidly. After a

¹ LOEB, J., *J. Gen. Physiol.*, vol. 4, p. 351, 1921-22.

number of hours the suspended particles were separated from the outside solution by filtration, the gelatin was melted and put into vessels with two bent tubes (see Fig. 71). After the gelatin had set to a gel (by cooling) the P.D. between the solid gel and the outside solution (filtrate) was determined with the electrometer. The P.D. was that of the following cell:

calomel electrode	saturated KCl	outside aqueous solution	solid gel	saturated KCl	calomel electrode
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Everything else being symmetrical, the P.D. measured was that between the suspended particles of gelatin (solid gel) and the outside solution with which the gelatin micellæ had been in complete or approximate equilibrium.

It can be shown that the membrane potential between the micellæ and the surrounding solution is influenced in the same way by pH, valency, and salts as is the P.D. between a protein solution and an aqueous solution separated from each other by a collodion membrane. It was then necessary to ascertain whether this P.D. was due to a Donnan equilibrium, and for this purpose the pH inside of the (melted) gelatin micellæ and the pH of the outside solution were measured with the hydrogen electrode. It was found that the hydrogen electrode potentials agreed as closely with the membrane potentials observed with indifferent electrodes as the accuracy of the measurements permitted. This makes it highly probable that the membrane potential of a gel is determined by the Donnan equilibrium. The accuracy of the P.D. measurements is not as great as in the case of the experiments of the preceding chapter, for reasons which we have not yet been able to ascertain.

2. The Influence of pH on Membrane Potentials of Gelatin Gels

One-gram samples of powdered isoelectric gelatin going through mesh 30, but not through mesh 60, were put into 350 c.c. of water containing various quantities of HCl (see first horizontal row of Table XXXVII), and left in these solutions for 24 hours at 20°C.

TABLE XXXVII.—INFLUENCE OF ACID ON SWELLING AND MEMBRANE POTENTIALS OF GELS

Cubic centimeters 0.1 \times HCl in 100 c.c. of H ₂ O	0.5	1	2	4	6	8	10	12	15	20	30	40
Relative volume of gel.....	30	40	62	73	75	73	66	64	54	50	41	37
pH of melted gelatin (inside).....	4.58	4.27	3.76	3.26	2.92	2.57	2.41	2.29	2.11	1.96	1.78	1.59
pH of supernatant liquid (outside).....	3.89	3.45	3.04	2.65	2.42	2.27	2.16	2.07	1.85	1.82	1.63	1.49
pH inside minus pH outside.....	0.69	0.82	0.72	0.61	0.48	0.30	0.25	0.22	0.16	0.14	0.15	0.10
Hydrogen electrode P.D. (millivolts).....	+40.7	+48.4	+42.5	+36.0	+28.4	+17.7	+14.7	+13.0	+9.5	+8.3	+7.7	+5.9
Membrane P.D. (millivolts).....	+37.5	+39.0	+38.0	+29.5	+22.0	+17.7	+17.7	+18.2	+17.0	+10.7	+8.6	+5.4

TABLE XXXVIII.—INFLUENCE OF ACID AND ALKALI ON SWELLING AND MEMBRANE POTENTIALS OF GELS

Cubic centimeters 0.1 N HCl or NaOH in a 100-c.c. solution	HCl				NaOH							
	HCl				NaOH							
	1.0	0.5	0.2	0.1	0	0.1	0.2	0.5	1.0	2.0	4.0	
Relative volume of gelatin.....	28	20	18	16	17	18	28	37	40	47	48	
pH of melted gelatin (inside).....	4.44	4.56	4.79	4.85	4.89	4.98	5.06	5.50	6.74	9.54	10.15	
pH of supernatant liquid (outside).....	3.35	3.55	3.92	4.24	4.97	5.96	6.24	6.46	7.30	11.08	11.08	
pH inside minus pH outside.....	+1.09	+1.01	+0.87	+0.61	0.08	-0.98	-1.18	-0.96	-0.56	-0.02	-0.93	
Hydrogen electrode P.D. (millivolts).....	+63.0	+38.6	+51.0	+36.0	-4.5	-57.0	-68.0	-56.0	-33.0	-59.0	-48.0	
Membrane P.D. (millivolts).....	+56.0	+55.5	+36.5	+15.0	-17.5	-59.0	-61.0	-70.0	-66.0	-46.0	-36.0	

The mixtures were occasionally stirred. After 24 hours the relative volume of the particles was measured and they were put on a filter to allow the acid to drain off. The gelatin was then melted by heating to 45°C. and poured into glass cylinders and the P.D. between gelatin and aqueous solution was ascertained. One of the two glass tubes dipped into a beaker containing the outside HCl solution (the filtrate) with which the gelatin had been in equilibrium, and the other dipped into a beaker containing a saturated solution of KCl. Each beaker was connected with a calomel electrode (filled with saturated KCl) leading to a Comp-ton electrometer. The last row in Table XXXVII gives the observed P.D. in millivolts.

The pH of the melted gelatin was then determined with the hydrogen electrode. This is called pH inside in Table XXXVII. The pH of the outside solutions (filtrate) was determined in the same way.

Tables XXXVII and XXXVIII show that the error in the measurements is greater than it was in the case of the measurements of the membrane potentials between a gelatin solution inside a collodion bag and the outside solution. The majority of experiments in Table XXXVII show a good agreement between the membrane potentials of the gel and the hydrogen electrode potentials, except that occasionally an observation shows a greater divergence. The writer feels certain that these isolated divergent results are due to experimental error, which in the future will in all probability be discovered and eliminated. The same is true for the results in Table XXXVIII. On the whole, the results leave no doubt that in spite of these occasional individual errors the potential differences between the jelly and the surrounding liquid are determined by the Donnan equilibrium.

3. The Influence of Acid and Alkali on the Sign of Charge of Gelatin Gels

It was shown that a protein solution assumes a positive charge on the acid and a negative charge on the alkaline side of the isoelectric point. It can be shown that this is also true for the charges of the suspended particles of powdered gelatin, and that this change of sign of charge of these particles on going from the acid to the alkaline side of the isoelectric point is accompanied

by a change in the sign of the value (pH inside micellæ minus pH outside).

One gram of powdered gelatin of grain size between mesh 30 and 60, rendered isoelectric, was put into each of a series of closed flasks containing 350 c.c. of distilled water with varying quantities of 0.1 N HCl or NaOH per 100 c.c. (see Table XXXVIII). The temperature was 20°C . After 4 hours the powdered gelatin was separated from its supernatant liquid by filtration, the gelatin was melted, and the pH of the melted gelatin and of the outside solution (filtrate) were measured. The gelatin was then solidified and the P.D. between the solid gelatin and the filtrate (outside solution) determined, as described. The results of the experiments are given in Table XXXVIII. The first row gives the number of cubic centimeters of 0.1 N HCl or NaOH contained originally in 100-c.c. outside solution. The next row gives the relative volume of the solid mass of gelatin, *i.e.*, the degree of swelling. The rest of the table needs no explanation. It is obvious that pH inside minus pH outside is positive as long as the pH of the gelatin is on the acid side of the isoelectric point, while it is negative when the gelatin is on the alkaline side of the isoelectric point. The turning point is approximately at the isoelectric point, but the measurements near the isoelectric point are obviously vitiated by experimental errors and possibly by some other factor, so that we cannot demonstrate more by the experiment than that a jelly of metal gelatinate has the opposite sign of charge to that of a jelly of gelatin chloride, and that this difference is accompanied by a reversal of the sign of the value of pH inside minus pH outside, which is positive in the case of gelatin chloride and negative in the case of Na gelatinate. It may also be pointed out that the minimum of swelling (volume) coincides with the minimum of P.D.

4. The Influence of Salts on Membrane Potentials of Solid Jellies

The most important fact which a theory of the electrical charge of solid gels is expected to explain is the annihilation of these charges by neutral salts. Those who believe in the adsorption theory assume that both ions of a neutral salt are adsorbed by the colloidal particles, and that the salt ion with the opposite

sign of charge to that of the colloidal particle diminishes the charge of the latter, while the salt ion with the same sign of charge as the colloidal particle increases the charge of the latter, and the more the higher the valency of the ion of the salt. The idea that such an adsorption occurs is definitely refuted by the experiments discussed in Chap. II (see Figs. 1 and 2). It might, however, be possible that the ion with the same sign of charge as the colloidal particle might increase the charge of the colloidal particle in some other way than through adsorption, and it was necessary to test this possibility, which has found acceptance on the part of some chemists. The writer's experiments on anomalous osmosis have shown that when a dilute solution of a salt is separated from pure water by a collodion membrane coated with gelatin, if the salt solution and the water are brought to the same pH by adding an acid, *e.g.*, HNO_3 , the potential difference on the two opposite sides of the membrane increases with the valency of the cation of the salt used, *i.e.*, in the order

$$\text{Ce} > \text{Ca} > \text{Na}.$$

This influence was found to be due to a diffusion potential.¹ Nevertheless it seemed necessary to determine whether or not these cations influenced the charge of solid gels of gelatin chloride in the same way. If this were true, the depressing effect of CeCl_3 on the charge of the gel should be less than the depressing effect of CaCl_2 , and the depressing effect of CaCl_2 should be less than the depressing effect of NaCl , provided the pH is on the acid side of the isoelectric point.

If, on the other hand, the Donnan effect alone determines the depressing effect of the salt on the charge of the solid gels of gelatin, this depressing effect should be exclusively due to the anion of the salt on the acid side of the isoelectric point of the gelatin, while the cation of the salt should have no effect. This follows from the discussion in the preceding chapter, according to which the membrane potential between gelatin chloride solution and water is determined by the value of $\log(1 + \frac{z}{y})$, where z and y are the anions. The cations do not enter into the term on the acid side of the isoelectric point. We shall see that the

¹ LOEB, J., *J. Gen. Physiol.*, vol. 4, pp. 213, 463, 1921-22.

measurements of the P.D. between gel and surrounding solution are sufficiently accurate to leave no doubt that the Donnan equilibrium alone determines the charge of the gel and that the cation of the salt does not increase the charge of the gel of gelatin chloride.

In order to get accurate measurements it was necessary to use micellæ of gelatin chloride of a pH sufficiently far from the isoelectric point to avoid the errors of the measurements which occur near that point. We weighed out doses of 1 gm. of powdered gelatin of a pH of near 7.0 and made them isoelectric by treatment with $M/128$ acetic acid and subsequent washing, as described in Chap. II. In this process some gelatin was dissolved and lost (probably about 25 per cent). The isoelectric powdered gelatin was put into 200 c.c. of H_2O or a solution of different concentrations of a salt— $NaCl$, $CaCl_2$, $BaCl_2$, $CeCl_3$, or Na_2SO_4 —containing 16 c.c. of $0.1 N HCl$. This brought the pH of the gel down to 2.8 or less, as Tables XXXIX to XLIII show. The powdered gelatin was left in these acid-salt solutions for about 2 hours at $20^\circ C.$, with frequent stirring. Then the supernatant liquid was separated from the powdered particles of gelatin by filtration and the P.D. between the gel and the surrounding liquid (filtrate) measured with the Compton electrometer, using the electrodes described in Fig. 71. Tables XXXIX to XLIII give the results. The uppermost row gives the nature and concentration of the salt. The next row gives the relative volume of the gel of gelatin, and the depressing influence of the salt on the swelling; then follow the values for pH inside and outside measured with the hydrogen electrode and then the values pH inside minus pH outside. The last two columns give the hydrogen electrode potentials and the membrane P.D. observed with the Compton electrometer and the indifferent electrodes described in Fig. 71.

The fact in common to all the experiments is the satisfactory agreement between the two types of potential, except that the hydrogen electrode potential is on the average about 3 millivolts higher than the membrane potential and the cause for this difference is unknown at present. It is, however, a constant difference and has therefore no relation to the nature of the salt used.

The main fact is that the depressing effect of the four salts, $NaCl$, $CaCl_2$, $BaCl_2$, and $CeCl_3$, is determined by the chlorine ion

TABLE XXXIX.—INFLUENCE OF CONCENTRATION OF NaCl ON THE MEMBRANE POTENTIALS OF A JELLY OF GELATIN CHLORIDE

Concentration of NaCl											
	m/8,102	m/4,006	m/2,048	m/1,024	m/512	m/256	m/128	m/64	m/32	m/16	m/8
Relative volume of solid gelatin.....	49	47	46	45	44	40	40	38	29	25	25
pH inside.....	2.79	2.74	2.76	2.75	2.72	2.67	2.56	2.54	2.47	2.45	2.41
pH outside.....	2.28	2.28	2.28	2.28	2.28	2.29	2.29	2.31	2.33	2.33	2.34
pH inside minus pH outside.....	0.51	0.46	0.48	0.47	0.44	0.39	0.27	0.23	0.14	0.12	0.07
Hydrogen electrode P.D. (millivolts).....	+29.5	+27.8	+27.8	+27.0	+25.5	+22.6	+15.6	+13.3	+8.0	+7.0	+4.0
Membrane P.D. (millivolts).....	+25.5	+25.0	+25.0	+23.0	+21.5	+18.5	+14.0	+10.5	+7.0	+5.5	+2.5

TABLE XL.—INFLUENCE OF CONCENTRATION OF CaCl₂ ON THE MEMBRANE POTENTIALS OF A JELLY OF GELATIN CHLORIDE

Concentration of CaCl ₂											
	m/8,192	m/4,096	m/2,048	m/1,024	m/512	m/256	m/128	m/64	m/32	m/16	m/8
Relative volume of solid gelatin.....	50	48	44	42	40	38	33	30	28	22	20
pH inside.....	2.80	2.80	2.75	2.77	2.72	2.71	2.65	2.59	2.49	2.47	2.43
pH outside.....	2.29	2.29	2.30	2.29	2.30	2.32	2.33	2.34	2.36	2.36	2.38
pH inside minus pH outside.....	0.51	0.51	0.46	0.47	0.43	0.41	0.33	0.26	0.19	0.13	0.07
Hydrogen electrode P.D. (millivolts).....	+29.5	+26.7	+27.2	+25.0	+23.8	+19.1	+15.1	+11.0	+7.5	+5.2	+4.1
Membrane P.D. (millivolts).....	+25.5	+23.0	+24.0	+21.0	+19.0	+15.5	+11.5	+7.5	+5.0	+3.0	+2.5

TABLE XLI.—INFLUENCE OF CONCENTRATION OF BaCl_2 ON THE MEMBRANE POTENTIALS OF A JELLY OF GELATIN CHLORIDE

	Concentration of BaCl_2											
	0	M/8.102	M/4.096	M/2.048	M/1.024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
Relative volume of solid gelatin.....	51	51	46	45	41	41	41	34	31	25	24	22
pH inside.....	2.80	2.77	2.73	2.73	2.73	2.67	2.65	2.57	2.55	2.51	2.47	2.44
pH outside.....	2.31	2.28	2.28	2.28	2.29	2.29	2.30	*2.33	2.35	2.38	2.39	2.39
pH inside minus pH outside.....	0.49	0.49	0.45	0.45	0.44	0.38	0.35	0.24	0.20	0.13	0.08	0.05
Hydrogen electrode P.D. (millivolts).....	+28.5	+28.5	+26.0	+26.0	+25.5	+22.0	+20.0	+14.0	+11.5	+7.5	+4.5	+3.0
Membrane P.D. (millivolts).....	+26.0	+25.0	+24.0	+23.0	+22.0	+18.5	+15.0	+11.0	+7.5	+5.5	+2.5	+2.0

TABLE XLII.—INFLUENCE OF CONCENTRATION OF CeCl_3 ON THE MEMBRANE POTENTIALS OF A JELLY OF GELATIN CHLORIDE

Concentration of CeCl_3											
0	M/8.102	M/4.096	M/2.048	M/1.024	M/512	M/256	M/128	M/64	M/32	M/16	
51	50	48	46	43	42	42	35	32	25	24	
Relative volume of solid gelatin.....	2.79	2.74	2.73	2.69	2.65	2.57	2.53	2.48	2.44	2.39	
pH inside.....	2.31	2.29	2.29	2.28	2.32	2.33	2.35	2.35	2.38	2.36	
pH outside.....	0.48	0.49	0.45	0.41	0.33	0.24	0.18	0.13	0.06	0.03	
pH inside minus pH outside.....											
Hydrogen electrode P.D. (millivolts).....	+28.0	+26.0	+25.5	+23.8	+19.0	+14.0	+10.5	+7.5	+3.5	+1.8	
Membrane P.D. (millivolts).....	+26.0	+25.0	+22.0	+21.5	+19.0	+11.5	+8.0	+5.0	+2.5	+2.5	

TABLE XLIII.—INFLUENCE OF CONCENTRATION OF Na_2SO_4 ON THE MEMBRANE POTENTIALS OF A JELLY OF GELATIN CHLORIDE

	Concentration of Na_2SO_4											
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
Relative volume of solid gelatin.....	52	51	49	47	44	40	38	34	28	24	23	22
pH inside.....	2.80	2.75	2.75	2.74	2.69	2.68	2.66	2.66	2.68	2.75	2.83	2.92
pH outside.....	2.33	2.29	2.30	2.32	2.32	2.36	2.41	2.45	2.56	2.67	2.79	2.89
pH inside minus pH outside.....	0.47	0.46	0.45	0.42	0.37	0.32	0.25	0.21	0.12	0.08	0.04	0.03
Hydrogen electrode P.D. (millivolts).....	+27.3	+26.7	+26.1	+24.3	+21.5	+18.5	+14.5	+12.2	+7.0	+4.6	+2.3	+1.7
Membrane P.D. (millivolts).....	+25.0	+22.0	+22.5	+21.0	+18.0	+16.0	+11.5	+8.5	+6.5	+4.0	+3.0	+1.5

concentration, and that the valency of the cation has no influence. This leaves no doubt that the charge of a solid jelly of gelatin is an unequivocal function of the Donnan equilibrium.

The depressing action of Na_2SO_4 is about four times as great as that of NaCl (Fig. 72).

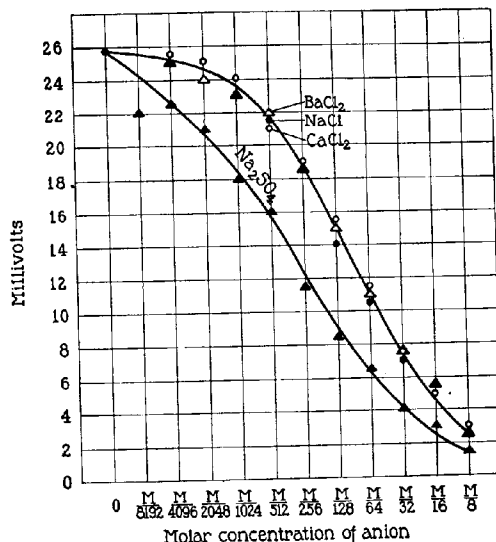


FIG. 72.—Depressing action of salts on the membrane potentials of a jelly of gelatin chloride.

II. PROCTER'S AND WILSON'S OSMOTIC THEORY OF SWELLING

We have already stated in the first part that in the large gelatin molecule we must distinguish between oily and aqueous groups. When gelatin is in aqueous solution two gelatin molecules can adhere to each other when their oily groups happen to come in contact, and in this way aggregates are formed. Since, however, this adhesion and aggregate formation need not and probably does not modify the forces of affinity between the aqueous groups and the water, the relative distance between the molecules or ~~jons~~ of gelatin in the solution will not be changed by the

adhesion. Finally all the molecules of gelatin will adhere and form a gel, provided first, that the concentration of gelatin makes this possible, and second, that the heat agitation, *i.e.*, the temperature, is low enough not to disrupt the cohesion of the molecules with their oily groups.

When a dry gel of isoelectric gelatin is put into water the forces of attraction between the water and the aqueous groups of the gelatin molecules of the gel will cause water to enter the gel. This is only a phenomenon of solution of water in the solid gelatin. Since the isoelectric gelatin is not ionized, the Donnan equilibrium does not act, and the swelling of isoelectric gelatin in water of pH 4.7 is due to causes different from those that were responsible for the influence of acids on swelling. When some acid or alkali is added to the water, ionizable gelatin salts are formed and this ionization leads to the establishment of a Donnan equilibrium between the gel and the outside solution, in which the osmotic pressure of the crystalloidal ions inside the solution becomes higher than that of the outside solution. This excess of the concentration of the crystalloidal ions inside the gel and outside due to the Donnan equilibrium causes the swelling due to acid. The measurements of the membrane equilibria in this case mentioned above leave no doubt about this fact.

Procter¹ and Procter and Wilson² applied Donnan's equilibrium theory to the explanation of the swelling of gelatin in acid. According to these authors, the force which causes the entrance of water, and hence the increase of volume in a solid block of gelatin in acid, is osmotic, and the opposing force which limits the swelling is the force of cohesion between the gelatin molecules or ions constituting the framework inside of which the water is occluded. These cohesive forces thereby play the same rôle in the swelling equilibrium as does the hydrostatic pressure on the membrane in the experiments on osmotic pressure.

The protein ions constituting a jelly of gelatin chloride cannot diffuse and hence, according to Procter and Wilson, can exercise no measurable osmotic pressure, while the chlorine anions in combination with them are retained in the jelly by the electrostatic attraction of the gelatin ion but exert osmotic pressure.

¹ PROCTER, H. R., *J. Chem. Soc.*, vol. 105, p. 313, 1914.

² PROCTER, H. R. and WILSON, J. A., *J. Chem. Soc.*, vol. 109, p. 307, 1916.

This difference in the diffusibility of the two opposite ions of the gelatin chloride gives rise to the condition leading to the establishment of Donnan's membrane equilibrium. If x be the concentration of the H and Cl ions in the outside solution, y the concentration of the free H and Cl ions in the solid gel, and z the concentration of Cl ions in combination with gelatin, the Donnan equilibrium is expressed by the equation

$$x^2 = y(y + z)$$

and the osmotic force e for the absorption of water by the gel is

$$e = 2y + z - 2x.$$

The reader will notice that this is the formula applied later by the writer to osmotic pressure and discussed in Chap. XII.

J. A. and W. H. Wilson¹ developed Procter's line of reasoning further and derived the following formula by purely mathematical reasoning from the assumption that gelatin combines chemically with HCl to form a highly ionizable gelatin chloride:

$$V(K + y)(CV + 2\sqrt{CVy}) - y = 0,$$

where V is the increase in volume in cubic centimeters of one milliequivalent weight of gelatin, C is the constant corresponding to the modulus of elasticity of the gelatin, and K is a constant defined by the equation

$$[\text{gelatin}] [\text{H}^+] = K[\text{gelatin ion}].$$

Given the constants, it is obviously possible to calculate all the variables of the equilibrium.

Procter and Wilson found the value $K = 0.00015$ by means of the hydrogen electrode on gelatin solutions and the value $C = 0.0003$ at 18°C. from experiments on the swelling of gelatin jellies. From Procter's data on gelatin, Wilson² calculated 768 as its equivalent weight. This value is lower than that found by Hitchcock,³ which was 1,120, as stated in Chap. IV, p. 71. Using these constants, Wilson and Wilson calculated the variables V , y , and z for comparison with the data obtained experimentally

¹ WILSON, J. A. and WILSON, W. H., *J. Am. Chem. Soc.*, vol. 40, p. 886, 1918.

² WILSON, J. A., *J. Am. Leather Chem. Assoc.*, vol. 12, p. 108, 1917.

³ HITCHCOCK, D. I., *J. Gen. Physiol.*, vol. 4, p. 733, 1921-22; vol. 6, p. 95, 1923-24.

by Procter. The calculated and observed results are shown in Table XLIV and it will be seen that the agreement is absolute, within the limits of Procter's experimental error. This is shown even more strikingly when the values are plotted. Procter and Wilson regard this as establishing their theory quantitatively.

The relation of V to e is governed by Hooke's law, *ut tensio sicvis*, and since e represents a pressure equal in all directions, the result is a pull upon the jelly equal in each dimension. The quantitative expression is

$$e = CV,$$

where the constant C is determined by the bulk modulus of the gelatin.

TABLE XLIV¹

x	V		y		z	
	Calculated	Observed	Calculated	Observed	Calculated	Observed
0.0032	43.2	41.2	0.0005	0.0005	0.018	0.017
0.0073	40.8	44.5	0.002	0.002	0.022	0.018
0.0077	40.2	40.1	0.002	0.002	0.023	0.020
0.0120	37.5	39.9	0.005	0.006	0.026	0.021
0.0122	37.3	39.7	0.005	0.006	0.026	0.021
0.0170	34.5	31.1	0.008	0.009	0.028	0.028
0.0172	34.3	37.0	0.008	0.009	0.028	0.022
0.0406	26.7	28.0	0.026	0.030	0.037	0.031
0.0420	26.4	23.4	0.027	0.030	0.038	0.038
0.0576	24.0	26.1	0.041	0.043	0.041	0.036
0.0666	23.0	21.4	0.049	0.050	0.043	0.045
0.0680	22.8	22.4	0.050	0.053	0.044	0.039
0.0930	20.7	17.7	0.072	0.072	0.049	0.054
0.0944	20.5	20.3	0.073	0.072	0.049	0.049
0.1052	19.8	22.9	0.083	0.085	0.051	0.043
0.1160	18.9	18.7	0.095	0.090	0.053	0.058
0.1434	17.9	18.4	0.118	0.118	0.056	0.055
0.1435	17.9	18.6	0.118	0.118	0.056	0.054
0.1685	17.1	18.0	0.141	0.138	0.059	0.062
0.1925	16.3	15.8	0.164	0.161	0.061	0.068
0.1940	16.2	17.4	0.166	0.165	0.061	0.060
0.1945	16.2	17.0	0.167	0.164	0.061	0.062

¹ Observed values are taken from PROCTER, H. R., *J. Chem. Soc.*, vol. 105, p. 313, 1914. The observed value for V given in this table is the increase in volume in cubic centimeters of 0.768 gm. of gelatin. Values for x , y , and z are given in mols per liter.

Procter and Wilson then explain on the basis of the Donnan equation why the value of e , and therefore also V , should follow

a curve of the particular type it does. By proper substitution from the thermodynamic and osmotic equations it follows that:

$$e = -2x + \sqrt{4x^2 + z^2}.$$

As the concentration of acid is increased from zero to some small, but finite, value, z must necessarily increase at a very much greater rate than x . This is shown very markedly in the most dilute solutions, where almost all the acid added combines with the gelatin; but z has a limiting value, which is determined by the total concentration of gelatin with which we started. Now z must either approach this limiting value or diminish, which it would do if the ionization of the gelatin chloride were sufficiently repressed. In either case:

$$\lim_{x = \infty} \sqrt{4x^2 + z^2} = \sqrt{4x^2}$$

from which it follows that:

$$\lim_{x = \infty} e = -2x + 2x = 0.$$

It is clear from this that, as x increases from zero, e must increase to a maximum and then decrease, approaching zero asymptotically, regardless of whether or not the ionization of the gelatin salt is appreciably repressed.¹

As far as the depressing action of salt on swelling is concerned, Procter and Wilson do not accept the idea that it is due to the repression of ionization.

Whilst the salt undoubtedly represses the ionization of the gelatin chloride to some extent, it would scarcely be sufficient to account for the fact that salt reduces the volume of jelly almost to that of dry gelatin. The chief action is probably that the addition of salt corresponds with an increase in the value of x , and that this increase in x must, according to the equation just discussed, produce a decrease in the value of e , with a corresponding diminution of the volume of the jelly.¹

There can be little doubt that the osmotic theory of Procter and Wilson accounts quantitatively for the process of swelling; no other theory has thus far been offered which can claim the same result.

The force which opposes and limits the swelling is the cohesion between the molecules or ions constituting the gel. When this force is diminished the swelling should increase. Procter and

¹ PROCTER, H. R. and WILSON, J. A., *J. Chem. Soc.*, vol. 109, p. 317, 1916.

Wilson have pointed out that this is the case since the swelling of gelatin increases when the gel is heated.¹

The forces of cohesion depend not only on temperature but also on chemical constitution. They are forces of the same kind as the forces determining solution; and it is well known that, *e.g.*, the substitution of Na for H in oleic acid increases the solubility of the substance in water, and that the substitution of K for Na increases the solubility still more. We might *a priori* expect that the forces of cohesion in a solid jelly of gelatin would also change considerably with the nature of the ion in combination with the gelatin. This is, however, as a rule, not the case. Only the valency, but not the nature of the ion in combination with gelatin, influences the swelling of gelatin. Thus, at the same temperature, at the same pH, and the same concentration of originally isoelectric gelatin, the swelling of gelatin chloride, nitrate, bromide, iodide, rhodanate, acetate, etc., is approximately the same, while that of gelatin sulphate and of sulphosalicylate is considerably lower. The swelling of Li, Na, K, and NH_4 gelatinates is also practically the same at the same pH and the same concentration of originally isoelectric gelatin, but the swelling of Mg, Ca, and Ba gelatinates is considerably less (see Chap. VII).

It was shown in Chap. VII that the same valency effect which exists in regard to osmotic pressure exists also in regard to swelling, and the theoretical discussion given in the preceding chapter for this valency effect in the case of osmotic pressure covers also the similar effect in the case of swelling.

In the case of casein-acid salts, which are less soluble than gelatin-acid salts, the nature of the anion is not without influence on the cohesive forces. Thus casein trichloracetate is practically as insoluble as casein sulphate, and neither of the two salts is capable of swelling, while the more soluble casein chloride and casein phosphate are capable of so doing. In the latter case the valency rule also holds, since the degree of swelling is practically the same for casein phosphate and casein chloride, at the same pH, temperature, and concentration of originally isoelectric casein.² The valency rule holds wherever colloidal behavior is concerned, since colloidal behavior is only the consequence of the

¹ PROCTER, H. R. and WILSON, J. A., *J. Chem. Soc.*, vol. 109, p. 315, 1916.

² LOEB, J. and LOEB, R. F., *J. Gen. Physiol.*, vol. 4, p. 187, 1921-22.

Donnan equilibrium and the equilibrium equation is only concerned with the sign and valency of the ion. The problems of solubility and of cohesion have only an indirect connection with colloidal behavior, and the fact that solubility and cohesion depend upon the specific nature of the ion (in addition to its sign of charge and valency) is not in conflict with the other fact that in the truly colloidal phenomena only the sign of charge and valency of an ion are concerned.

In a gel of gelatin at a pH different from that of the isoelectric point, part of the water is held by the forces of chemical attraction between the aqueous groups of gelatin and water; and part is held by the osmotic pressure due to the excess of the concentration of crystalloidal ions inside the gel over the concentration outside. The influence of the pH and of the valency of the anion of the acid or the cation of the alkali on this osmotic pressure must be the same as that on the osmotic pressure of a protein in solution, except that the force which limits the swelling of the gel is the cohesion between the protein molecules, while the force which limits the attraction of the protein solution for water is the hydrostatic pressure of the solution. We understand why the influence of acids and alkalies on swelling is a similar function of the pH and valency of ions as the influence on the osmotic pressure. We also understand why salts depress the swelling, since they depress the difference in the concentration of diffusible crystalloidal ions inside and outside the gel. This is shown by the pH measurements of the gel and the outside solution and this difference is similar to that of the osmotic pressure experiments with protein solutions. The mystery of the analogous effect of electrolytes on osmotic pressure of protein solutions and the swelling of gels thus finds a very simple explanation.

The Hofmeister ion series have been developed on the basis of experiments on swelling.¹ Hofmeister put pieces of solid gelatin in solutions of various substances, salts, and non-electrolytes, of very high concentration, and noticed that the increase in weight of the gelatin plates varied according to the nature of the substance. According to their relative efficiency to increase the swelling, the substances were arranged in the following series, which was the beginning of the Hofmeister series:

¹ HOFMEISTER, F., *Arch. expil. Path. Pharmacol.*, vol. 28, p. 210, 1890-91.

Sodium sulphate, tartrate, and citrate.

Sodium acetate, grape sugar, cane sugar.

Water.

NaCl, KCl, NH_4Cl .

NaClO_3 , NaNO_3 , NaBr.

The lowest concentration of these solutions was $\text{M}/2$ and went up to 4 M. It is hardly necessary to point out that the effects observed by Hofmeister have nothing to do with the production of swelling by acid and alkali and with the depression of such swelling by salts, since the swelling of gelatin caused by acid and alkali is already completely annihilated by concentrations of salts of less than $\text{M}/2$. Further, salts cause in such cases only a depression, not an increase in swelling. It is obvious that the effects observed by Hofmeister have no connection with the salt effects dependent on the Donnan equilibrium.

Stiasny and Ackermann¹ observed that dry powder of skin swells more in high concentrations of salts than in water. In this case the effect of the high concentrations of salts consisted probably in an increase in the solubility and a decrease in the cohesion between the molecules of solid collagen, whereby water was able to diffuse into this otherwise impermeable material.

It is obvious that both in Hofmeister's and Stiasny's experiments the salts acted on different physical properties from those in the Donnan equilibrium. It is unfortunate that the word "swelling" is applied indiscriminately to changes in volume of a solid in water, regardless of the fact that the forces leading to these changes may be entirely different. When these differences in the nature of these forces are taken into consideration, it becomes obvious why only the valency rule holds where swelling depends on the Donnan equilibrium, while in addition the chemical nature of the ions may play a rôle when the swelling is due to variations of crystalloidal forces, such as forces of cohesion or solubility.

¹ STIASNY, E. and ACKERMANN, W., *Kolloidchem. Beihfte*, vol. 17, p. 219, 1923.

CHAPTER XIV

VISCOSITY¹

1. It will make it easier, perhaps, for the reader to understand the two following chapters if it is pointed out at the beginning that there are two kinds of viscosity: one found in all solutions of crystalloids and colloids alike, which has no connection with colloidal behavior or the Donnan equilibrium; and a second type of viscosity which is due to the swelling of submicroscopic solid particles in a solution. Only this latter type of viscosity is specific for colloidal phenomena and depends, as will be shown, on the Donnan equilibrium. The latter colloidal type of viscosity is of a much greater order of magnitude than the former crystalloidal one, which may therefore be neglected in the following discussion.

We have seen in Chaps. VII and VIII that the influence of electrolytes on the viscosity of the solutions of certain proteins, *e.g.*, gelatin or casein, is similar to the influence of electrolytes on osmotic pressure, swelling, and potential differences. The explanation given for the influence of electrolytes on the last-named properties was based on the theory of Donnan's membrane equilibrium. This theory can only be applied where the diffusion of one type of ions is prevented, while no such block exists for other ions. In the experiments on osmotic pressure or P.D. of protein solutions the collodion membrane permits the diffusion of crystalloidal ions while preventing the diffusion of the protein ions; and in the case of the solid gel the protein ions are prevented by the forces of cohesion from diffusing into the surrounding solution free from protein. But this raises the problem of how the Donnan equilibrium can be applied to the viscosity of protein solutions. We intend to show that the answer lies in the fact that, although protein solutions may be and probably are primarily

¹ LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 827, 1920-21; vol. 4, pp. 73, 97, 1921-22.

true solutions consisting of isolated protein ions and molecules distributed equally through the water, they contain under certain conditions submicroscopic solid particles of protein. We shall see that the viscosity of protein solutions is only influenced in the same way by electrolytes as is the osmotic pressure when such solid protein particles are present in considerable numbers and when they are capable of swelling. If they are absent or scarce, or cannot swell, electrolytes will not influence the viscosity of protein solutions in the same way as electrolytes influence the osmotic pressure or the P.D. of protein solutions or the swelling of gels. In the following discussion we shall measure the viscosity of protein solutions by the time of outflow through a capillary tube, as described by Ostwald, and the quotient of this time over the time of outflow of pure water through the same viscometer at the same temperature will be referred to as the relative viscosity or as the viscosity ratio of the protein solution. This method of measuring the relative viscosity will require improvement, but it suffices for an approximate test of the validity of the theory.

Einstein¹ has developed a theory of the viscosity of solutions which makes the viscosity a linear function of the relative volume occupied by the solute in the solution

$$\eta = \eta_0(1 + 2.5\varphi), \quad (1)$$

where η_0 is the viscosity of the water at the temperature of the experiment, η the viscosity of the solution, and φ the fraction of the volume occupied by the solute in the volume of the solution. As Einstein points out, this formula can only be used when φ is very small and when the particles of the solute are spherical and large in comparison with the molecules of the solvent. This condition is no longer fulfilled in protein solutions when the relative volume occupied by the protein in the solution becomes too large.

Several authors have tried to modify Einstein's formula in order to make it applicable to higher concentrations of protein solutions. Hatschek² proposed to replace the constant 2.5 of Einstein's formula by the constant 4.5, but his deductions have

¹ EINSTEIN, A., *Ann. Physik*, vol. 19, p. 289, 1906; vol. 34, p. 591, 1911.

² HATSCHKE, E., *Kolloid-Z.*, vol. 11, p. 280, 1912.

been criticized both by Smoluchowski¹ and by Arrhenius.² Arrhenius has shown that a logarithmic formula, which he derives very ingeniously from Einstein's formula, fits the actual observations in a satisfactory way, this formula being

$$\log \eta - \log \eta_0 = \vartheta \varphi, \quad (2)$$

where φ is again the fraction of volume occupied by the protein in solution, ϑ a constant, while η and η_0 have the same significance as in Einstein's equation. We shall make use of Arrhenius's equation (2) when we are dealing with higher viscosities.

The formulæ of both Einstein and of Arrhenius make the viscosity a function of the relative volume occupied by the solute in the solution, and it must be our task to correlate the influence of electrolytes on viscosity with corresponding variations of the volume of the protein in solution.

The question then arises: How can the same mass of protein particles in solution change its relative volume under the influence of electrolytes? This is only possible if the relative volume occupied by the protein in the solution is increased by water shifting from solvent to solute. We therefore have to find out whether or not a shifting of water from the solvent to the solute is possible, so that the volume of the solvent is diminished and that of the solute increased. It is generally assumed that the mechanism for such a transfer of water from solvent to solute is explained by Pauli's hydration theory, which has been repeatedly referred to in this volume. Pauli suggested that the ionized molecule of protein is surrounded by a shell of water which is lacking in the non-ionized molecule. When protein is ionized, *i.e.*, by the addition of acid or alkali to isoelectric protein, a shell of water is formed around each individual protein ion. On this basis we can understand why the viscosity of a solution of isoelectric protein should increase with the addition of acid or alkali. The work of Lorenz, Born, and others, however, casts a doubt on the assumption of a general hydration of polyatomic ions. There are still other facts which show that the mere

¹ SMOLUCHOWSKI, M., *Kolloid-Z.*, vol. 18, p. 190, 1916.

² ARRHENIUS, S., *Meddelanden K. Vetenskapsakademiens Nobelinstitut*, vol. 3, No. 21, 1917.

ionization and consequent hydration of the individual protein ions cannot well be the cause of the influence of the pH on the relative viscosity of gelatin solutions.

Gelatin solutions show the characteristic influence of the pH on their viscosity, as is demonstrated in Fig. 73. The viscosity of gelatin solutions behaves qualitatively as we might expect on the basis of Pauli's hydration theory, except that the maximum of the viscosity lies at a pH higher than that for the maximum of ionization. If hydration of the individual protein ions were the cause of the variation of the viscosity of gelatin solutions, a

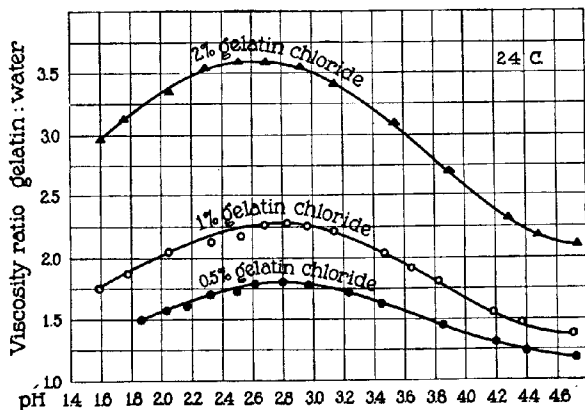


FIG. 73.—Influence of pH on viscosity of freshly prepared gelatin chloride solutions.

variation of the hydrogen ion concentration should have a similar influence on the viscosity of solutions of simple amino-acids, like glycocoll and alanine, to that which it has on the viscosity of gelatin solutions. Five per cent solutions of glycocoll and alanine were brought to different pH, from 5.0 to 2.0 and below, by the addition of HCl. Miss Brakeley found, in the writer's laboratory, that the variation of the pH of 5 per cent solutions of these two amino-acids between the limits of 5.0 and 1.16 had no measurable influence on the viscosity of the solution. G. Hedestrand¹ found in Euler's laboratory a slight variation in the

¹ HEDESTRAND, G., *Z. anorg. allgem. Chem.*, vol. 124, p. 153, 1922.

viscosity of 2 N glyocoll solutions upon the addition of acid or alkali; the minimum was found at pH 6.4, where the viscosity was about 1.36, while at pH 3.0 it was 1.38. This is an influence of pH of a considerably lower order of magnitude than the one found in the case of gelatin or casein solutions.

In the writer's experiments the viscosity of a 1 per cent solution of gelatin could easily be raised from 1.2 to 3.0 or 4.0 by the addition of acid. A 1 per cent solution of gelatin is at most 1/1,200 molar; more probably, perhaps, $M/2,400$. Hence, the effect of acid on the viscosity of gelatin solutions is more than a thousand times greater than that on solutions of amino-acids. This leaves no doubt that the influence of acid on viscosity has an entirely different cause in the two cases.

That the viscosity of amino-acids is a minimum at their isoelectric point is due probably to the fact that the ionization of the amino-acids is a minimum at the isoelectric point, but the influence of ionization on the viscosity of amino-acids has no connection with the Donnan effect, while the influence of acid on the second type of viscosity of gelatin solutions is due to the swelling of submicroscopic particles of solid jelly contained in the gelatin solution.

It is of importance to bear in mind that all the properties of proteins may be a minimum at the isoelectric point which depends on ionization, but the effect of ionization may be direct or indirect. It is direct in the case of solubility, and a certain type of viscosity—which we will call ordinary viscosity—possibly also in the case of surface tension; it is indirect in the case of membrane potentials, osmotic pressure, swelling, and that type of viscosity which is a function of swelling of suspended particles.

These results cast a serious doubt on the assumption that the variations in the curve of the viscosity of gelatin, as expressed in Fig. 73, were caused by variations in the hydration of the individual gelatin ions.

This doubt was increased by experiments on the influence of pH on the viscosity of crystalline egg albumin, which indicated only a slight, almost negligible influence of the pH on the viscosity. Figure 74 gives such an experiment with 3 per cent originally isoelectric albumin brought to different pH through the addition of HCl. The ordinates are the viscosity ratios of albumin

solution over water, drawn on a larger scale than those in Fig. 73, and the abscissæ are the pH of the solution. It is obvious that if compared with the gelatin curves, the pH has only a very slight influence on the viscosity of solutions of crystalline egg albumin between pH 4.6 and pH 1.0. With a further lowering of pH the viscosity suddenly rises, a fact to which we shall return later.

The method of the experiments was as follows: 50-c.c. samples of a 6 per cent solution of isoelectric crystalline egg albumin were mixed with 50 c.c. of HCl solutions of different concentrations and the pH measured. The solutions were rapidly brought to a

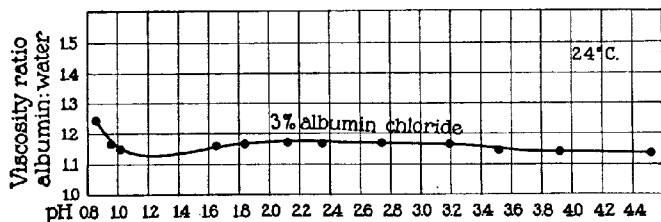


FIG. 74.—Showing that solutions of crystalline egg albumin have a low viscosity in comparison with gelatin solutions, and that the pH has little influence on the viscosity of solutions of crystalline egg albumin at pH over 1.0 and at ordinary temperature.

temperature of 24°C. and the viscosity was measured immediately at that temperature.

The question then arises: Why do amino-acids and at least one protein, namely, crystalline egg albumin, behave so differently from gelatin in regard to the influence of the pH on the viscosity of their solutions? As long as we assume that the influence of the hydrogen ion concentration on the viscosity of gelatin-acid salt solution is due to the hydration of the individual protein ions, this difference is incomprehensible, since the amino-acids as well as crystalline egg albumin should in this case show the same influence of ionization on viscosity as the gelatin.

The puzzle becomes still greater if we take into consideration the fact that the osmotic pressure of solutions of crystalline egg albumin is affected in the same way by the hydrogen ion concentration as is the osmotic pressure of gelatin solutions (Chap.

VII). Why, then, do these two proteins behave so differently as regards the influence of the pH on their viscosity?

We get an answer to this question by comparing the order of magnitude of the viscosity of solutions of crystalline egg albumin and of gelatin. The viscosity of solutions of crystalline egg albumin has a comparatively low order of magnitude if compared with the viscosity of solutions of gelatin of the same concentration of protein and the same pH. The viscosity of solutions of crystalline egg albumin of pH 5.1 (*i.e.*, near the isoelectric point) of concentrations from 1 to 14 per cent was measured at 15°C. (Fig. 75). The viscosity is not only low

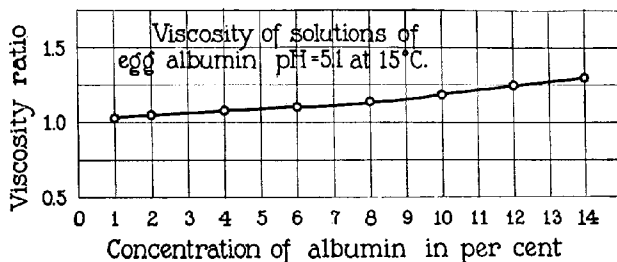


FIG. 75.—Viscosity ratio of solutions of crystalline egg albumin near the isoelectric point. Inside the concentrations used, the viscosity ratio is nearly a linear function of the concentration.

but it is also practically a linear function of the concentration. Figure 76 gives the viscosity of different concentrations of solutions of isoelectric gelatin at different temperatures. The solutions were prepared from the same stock solution of isoelectric gelatin and were rapidly heated to 45°C. and rapidly cooled to the desired temperature and then the time of outflow in an Ostwald viscometer was measured. This was done to avoid the increase in viscosity which occurs on standing and which is especially noticeable in the case of solutions of isoelectric gelatin. For the sake of conformity the same procedure was followed in the case of solutions of crystalline egg albumin. It is obvious that where the pH influences the viscosity in the same sense as the osmotic pressure, *e.g.*, in the case of gelatin solutions, the viscosity is of a much higher order of magnitude than where the pH has no such influ-

ence on viscosity as is the case in solutions of crystalline egg albumin.

It now remains to show that this difference in the order of magnitude of the viscosity of the two solutions is connected with the relative volume occupied by the protein in solution. The

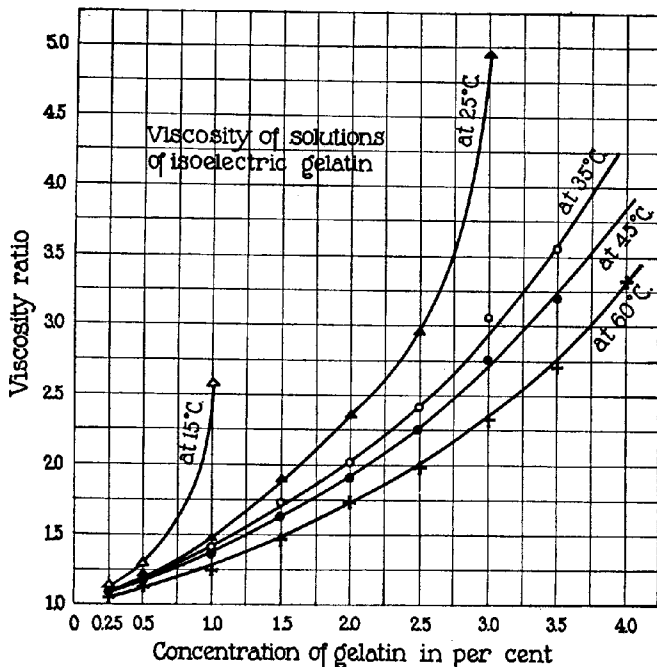


FIG. 76.—Influence of concentration on the viscosity of solutions of isoelectric gelatin.

low order of magnitude of the viscosity of solutions of crystalline egg albumin suggests a small relative volume; and, if this be true, the viscosity of solutions of crystalline egg albumin should obey the Einstein formula; while the high order of magnitude of viscosity of the solutions of gelatin suggests that a larger volume is occupied by the gelatin particles in solution and hence the constant 2.5 of Einstein's formula should be found too small; in other

words, the Einstein formula should be replaced by some other formula, *e.g.*, that of Arrhenius.

Einstein's formula is $\frac{\eta}{\eta_0} = 1 + 2.5\varphi$, where φ is the relative volume occupied by the protein in the solution, and $\frac{\eta}{\eta_0}$ is the viscosity ratio, *i.e.*, time of outflow of solution over time of outflow of water. The volume occupied by the protein in 100 c.c. of solution is

$$\varphi = \left(\frac{\eta}{\eta_0} - 1 \right) \frac{100}{2.5}.$$

By dividing the weight of albumin in solution by its volume we should obtain the density of albumin. Determinations of the density of albumin, by direct methods, give the value of 1.36 (Arrhenius). Table XLV shows that if we calculate the density

TABLE XLV

Concentration of crystalline egg albumin, per cent	$\frac{\eta}{\eta_0} - 1$	Calculated volume of albumin, cubic centimeters	Calculated density of albumin
14	0.290	11.6	1.20
12	0.240	9.6	1.25
10	0.185	7.4	1.35
8	0.132	5.3	1.51
6	0.100	4.0	1.50
4	0.074	2.96	1.36
2	0.042	1.7	1.17

of albumin on the basis of Einstein's formula, we obtain values which differ only inside the limits of experimental accuracy from the value 1.36 obtained by direct determination. The time of outflow of water through the viscometer was in this case 227 seconds at 15°C. These measurements show that the low order of magnitude of the viscosity of solutions of crystalline egg albumin is accompanied by a volume of albumin sufficiently low to permit the application of Einstein's formula, with the constant 2.5.

When we try to apply Einstein's formula in the same way to the viscosity measurements of isoelectric gelatin solutions, we find that the relative volume of gelatin in the solution and its density calculated on the basis of the constant 2.5 lead to impossible results. Thus the density of gelatin is probably not very different from that of egg albumin, *i.e.*, in the neighborhood of 1.4. The values calculated in Table XLVI with Einstein's viscosity constant 2.5. are from 20 to 40 times too low. Hence, the relative volume of gelatin in these solutions is far beyond the

TABLE XLVI

Concentration of isoelectric gelatin, per cent	$\frac{\eta}{\eta_0} - 1$ at 35°C.	Calculated volume of gelatin, cubic centimeters	Calculated density of gelatin
0.5	0.170	6.8	0.077
1.0	0.405	16.2	0.06
1.5	0.725	29.0	0.05
2.0	1.020	40.8	0.05
2.5	1.405	56.2	0.045
3.0	2.042	81.7	0.037
3.5	2.560	102.4	0.034

limit inside which the formula of Einstein is applicable. The formula of Arrhenius (2) leads to a fair agreement. According to this formula the logarithms of the viscosity ratio, when plotted over the concentration of the gelatin, should give a straight line. The agreement of the values for 45 and 35°C. with this theory is satisfactory (considering the limits of accuracy of the measurements), the logarithms of the viscosity increasing practically in direct proportion with the concentration (*i.e.*, the relative volume) of the gelatin in the solution (Table XLVII). At 60°C. the agreement is not quite so good, but still recognizable. At 25°C., however, it is satisfactory only at the lowest concentrations, but at the higher concentrations the viscosity grows more rapidly than the concentration. The reason for this is, however, obvious, since at this temperature the gelatin solution solidifies so rapidly that the viscosity measurements were no longer

possible for a concentration of 3.5 per cent gelatin solution, and for this reason the value of the viscosity of a 3 or a 2 per cent solution is already too high on account of the mechanical hindrance of the flow of the solution through the viscometer owing to partial solidification.

TABLE XLVII

Concentration of solution of iso-electric gelatin, per cent	$\log \frac{\eta}{\eta_0}$			
	60°C.	45°C.	35°C.	25°C.
0.25	0.0236	0.0306	0.0269	0.0374
0.5	0.0504	0.0682	0.0682	0.0792
1.0	0.0930	0.1350	0.1475	0.1685
1.5	0.1656	0.2135	0.2367	0.2765
2.0	0.2350	0.2796	0.3057	0.3701
2.5	0.2953	0.3512	0.3811	0.4691
3.0	0.3094	0.4409	0.4832	0.6941
3.5	0.4321	0.5051	0.5514	solidifies
4.0	0.5214	0.5660	0.6043	

These experiments lead to the following two conclusions:

1. Since the viscosity measurements of solutions of crystalline egg albumin and of gelatin agree fairly well with Einstein's and Arrhenius's formula respectively, it seems that the viscosity of solutions of proteins is primarily a function of the relative volume occupied by the protein in solution.

2. Since the measurements were made at (or near) the iso-electric point of the two proteins, the difference in the viscosity of solutions of gelatin and of crystalline egg albumin cannot be ascribed to differences in the degree of hydration of the individual protein ions, since at the isoelectric point the protein is practically not ionized.

It follows from these results that the difference in the order of magnitude of the viscosity of the two proteins must be due to the fact that gelatin possesses a mechanism for increasing its relative volume in solution which is lacking in the case of egg albumin (in not too high a concentration, at not too high a tem-

perature, and at a pH above 1.0), and this mechanism seems to be connected, in the case of gelatin solutions, with their tendency to set to a gel.

Zsigmondy states that Smoluchowski has explained the increase in the viscosity of a solution of aluminium oxide upon coagulation by the assumption of an occlusion of liquid between the particles. Smoluchowski calculates from the increase of viscosity during coagulation of aluminium oxide that the coagulating particles occupy a volume 400 to 500 times as great as that occupied by the dry material itself.¹ This apparent increase of volume he explains through the aggregation of needle-shaped particles, water being occluded between these particles. Smoluchowski apparently did not associate this occlusion of water with the Donnan equilibrium.

If we adopt this idea for the explanation of the high order of viscosity of gelatin solutions as compared with solutions of egg albumin, we reach the conclusion that the gelatin solutions contain submicroscopic particles of solid jelly, *i.e.*, micellæ which occlude relatively large quantities of water, whereby the relative volume occupied by the gelatin in solution is increased, and that such particles are lacking or scarce in the case of solutions of egg albumin. These submicroscopic particles of solid jelly are the precursors of the continuous jelly to which the gelatin solution has a tendency to set. The fact that these particles are lacking or scarce in the case of solutions of egg albumin is connected with the fact that the latter solutions have no tendency to set to a jelly at ordinary temperature and at pH above 1.0. When the pH is below 1.0 and the temperature higher the solutions of crystalline egg albumin set to a jelly, and in that case their viscosity becomes of the same high order of magnitude as that of gelatin solutions.

This assumption would also explain why the pH causes a similar variation in the viscosity of gelatin solutions as in their osmotic pressure, while the viscosity of solutions of crystalline egg albumin shows no such influence of the pH. There must arise a Donnan equilibrium between these submicroscopic

¹ Quoted from ZSIGMONDY R., "Kolloidchemie," 2d ed., p. 98, Leipzig, 1918. The paper of SMOLUCHOWSKI is inaccessible to the writer, since the number of the journal in which it appeared failed to reach the Institute during and since the war.

particles of solid jelly and the surrounding solution, and this Donnan equilibrium must regulate the amount of water occluded by the submicroscopic particles of solid jelly floating in the gelatin solution. Since the low order of magnitude of the viscosity of albumin solutions excludes the existence of a considerable number of such submicroscopic solid particles in the solution, it becomes obvious that the Donnan equilibrium cannot manifest itself to any large extent in the viscosity of solutions of this protein at not too high a concentration, at low temperatures, and at pH above 1.0.

2. It must then be our first task to find whether or not there exists a Donnan equilibrium between solid particles of gelatin and a surrounding weak solution of a gelatin salt, *e.g.*, gelatin chloride. The experiments were made by putting powdered gelatin in solutions of gelatin and then determining the membrane potentials and the hydrogen electrode potentials between the powdered particles of gelatin and the outside solutions. If there exists a Donnan equilibrium, the two potentials should agree.

At first thought it might seem strange that when solid granules of isoelectric gelatin are suspended in a solution of gelatin and HCl there should arise a difference in the distribution of the H and Cl ions inside the solid granules and the surrounding gelatin. Yet this is the case, as Table XLVIII shows, and the reason is easily understood. In the solid granules of gelatin the concentration of protein molecules is much higher than in the weak gelatin solution surrounding the granules, and if HCl is added the concentration of the gelatin ions must be higher inside the solid granules than in the dilute gelatin solution in which the granules are suspended. It follows from Donnan's theory that this difference in the concentration of protein ions inside the powdered particles and the solution must give rise to a membrane equilibrium, as a consequence of which a membrane potential must exist which agrees within the limits of accuracy of measurement with the hydrogen electrode potential.

The correctness of this expectation was confirmed by the following experiment: Mixtures of a solution of isoelectric gelatin and particles of powdered gelatin were made so that a 100-c.c. solution always contained 0.8 gm. of isoelectric gelatin in all. The proportion of solid and liquid gelatin varied, however, in

each case, as indicated in Table XLVIII. In each 100 c.c. of the mixture were contained 8 c.c. of 0.1 N HCl. The mixtures were kept for 2 hours at 20°C. and frequently agitated to accelerate the establishment of equilibrium between granules and solution. The solid powdered gelatin was then separated from the supernatant liquid by filtration.

In order to determine the membrane potential between these solid particles and the gelatin solutions, the solid particles were fused to a coherent gel in the vessels described for the measurements of the membrane potentials between gel and aqueous solution, except that in this case the potentials between the solid gel and the gelatin solution in which the powdered particles had been

TABLE XLVIII.—DONNAN EQUILIBRIUM BETWEEN PARTICLES OF POWDERED GELATIN AND GELATIN IN SOLUTION

Powdered gelatin in 100 c.c., gram	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Dissolved gelatin per 100 c.c., gram	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.0
pH of powdered gelatin	3.30	3.26	3.28	3.24	3.28	3.24	3.30	3.26
pH of supernatant gelatin solution	2.99	2.97	2.90	2.88	2.83	2.77	2.72	2.69	2.62
pH solid minus pH liquid gelatin	0.33	0.36	0.40	0.41	0.51	0.52	0.61	0.64
Hydrogen electrode P.D. (millivolts)	19.0	21.0	23.0	24.0	29.5	30.0	35.5	37.0
Membrane P.D. (millivolts)	14.5	17.0	17.0	17.5	23.0	26.0	30.0	33.0

suspended were measured. The values are found in Table XLVIII, showing that there exists a considerable membrane potential between the gelatin granules and weak solutions of gelatin, and that this P.D. increases with the relative increase in the concentration of solid gelatin, as was to be expected.

Measurements of the hydrogen electrode potentials showed that the membrane potentials were determined by the Donnan equilibrium,¹ though there exists, however, a constant discrepancy between the membrane potentials and the hydrogen electrode potentials which requires further investigation.

These results leave no doubt that the influence of acid on the viscosity of suspensions of powdered particles of gelatin in a

¹ The measurements of the P.D. between solid gelatin and solution are not as accurate as the measurements between liquids across a membrane.

gelatin solution is due to the swelling of these particles in accordance with the Donnan equilibrium.

3. It follows that a suspension of powdered gelatin in water should have a greater viscosity at a given temperature than if the same mass of gelatin were dissolved in water, since in the latter case part of the gelatin at least is in true solution (as we have seen in Part I) and this latter is incapable of increasing its volume by occluding water. It follows, furthermore, that the influence of electrolytes on the viscosity of suspensions of powdered gelatin should be the same as the influence of electrolytes on the osmotic pressure of gelatin solutions. It can be shown that both expectations are fulfilled.

Doses of 0.5 gm. of powdered gelatin were put into 100 c.c. of water containing 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12.5, 15, and 20 c.c. of 0.1 *N* HCl to bring the gelatin to different pH. The suspensions were allowed to stand 1 hour at 20°C. to bring about the swelling of the particles, and the viscosity of the suspensions was measured in a straight viscometer at 20°C. The time of outflow of water through the viscometer at 20°C. was 48.5 seconds. The upper curve in Fig. 77 gives the ratio of viscosity of suspensions to that of water at 20°C. (When the viscosity is high the values obtained are a little too great, owing to a gravity effect which causes the solid particles to collect above the upper opening of the capillary tube during a part of the time of the experiment, thus increasing temporarily the density of the suspension.) After the viscosity of a suspension was measured, the suspension was transformed into a solution by heating to 45°C. for 10 minutes; after that the solution was rapidly cooled to 20°C. and the viscosity of the gelatin solution was immediately measured with the same viscometer at 20°C. The lower curve in Fig. 77 shows that the viscosity was now considerably diminished. The abscissæ are the pH of the gelatin solutions. The effect of the pH on the viscosity of the powdered suspension of gelatin was very much greater than that observed in the case of gelatin solution, as becomes obvious by a comparison of Figs. 77 and 73. Immediately after melting and cooling to 20°C., this pH effect has almost completely disappeared, as is shown by a comparison of the lower with the upper curve in Fig. 77.

If we measure the volume of the suspended particles we find that it varies in a similar way as the viscosity. Samples of 0.5 gm. of Cooper's powdered commercial gelatin of a pH of about 6.0 were added to 100-c.c. portions of water containing varying amounts of HCl. The particles had uniform size (going through sieve 100 but not through sieve 120), but their shape was extremely irregular. They were left in the solution several hours

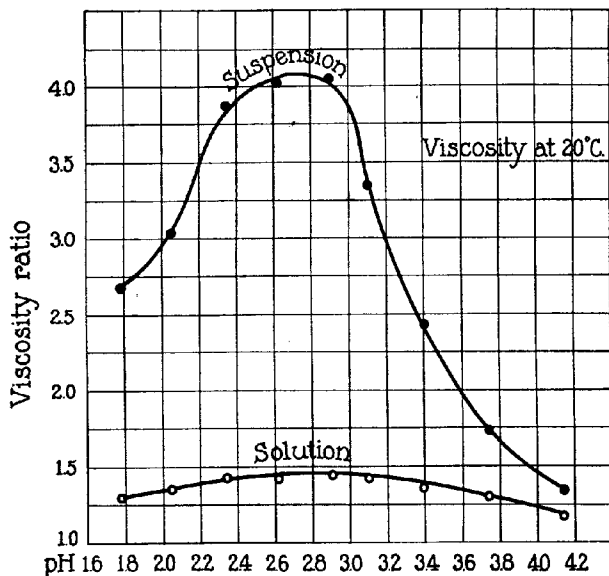


FIG. 77.—Difference in the viscosity of a suspension of 0.5 gm. of powdered gelatin in 100 c.c. and of the solution of the suspension in the same liquid; both viscosities were measured at 20°C.

at 20°C., and then their time of outflow through a capillary tube was ascertained at 20°C. The time of outflow of water through the viscometer at this temperature was 24 seconds. It was essential to stir the suspension thoroughly before sucking it into the viscometer, since the gelatin particles sink rapidly to the bottom of the dish.

After the viscosity measurements were taken the suspension was put on a filter of cotton wool and the supernatant water

allowed to drain off. By measuring the volume of the filtrate and deducting this from the original volume of the suspension (which was in all cases 100 c.c.), the volume of the gelatin was obtained (with a considerable error). Then the gelatin was melted and the pH of the melted mass of gelatin as well as of the filtrate was determined potentiometrically. Figure 78 gives the result of such an experiment. The lower curve shows the influence of the pH (of the gelatin) on the viscosity, and the upper curve the influence of the pH on the volume of the gelatin. The two curves are similar.

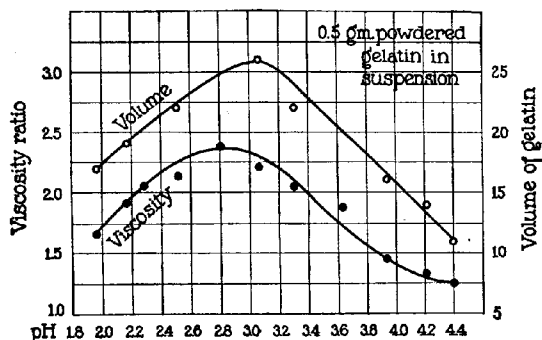


FIG. 78.—Showing that the influence of pH on viscosity of 0.5 per cent suspensions of powdered gelatin in water is similar to the influence of pH on viscosity of gelatin solutions, and that the volume occupied by the particles in the suspension varies in a similar way as the viscosity. Temperature 20°C.

The valency of the anion of the acid influences the viscosity of suspensions of protein in a similar way as it does the viscosity of solutions. This proof is furnished in Fig. 79. Doses of 0.5 gm. of finely powdered gelatin (going through a sieve of mesh size 100 but not through sieve of mesh size 120) of pH 7.0 were put into a series of beakers containing each 100 c.c. of HCl of different pH and kept in the solution overnight at a temperature of 20°C. Simultaneously a similar series of beakers containing each 100 c.c. of H_3PO_4 and H_2SO_4 of different pH (instead of HCl) were prepared, each receiving also 0.5 gm. of powdered gelatin. After 19 hours the viscosities of all these series of suspensions were determined at 20°C. Figure 79 gives the result, the ordinates

being the values for the viscosity ratios, gelatin suspension: water, and the abscissæ are the pH of the gelatin particles at equilibrium. The curves show that the viscosity of suspensions of gelatin sulphate is a little less than half that of suspensions of gelatin chloride and phosphate of the same pH. The curves for the suspensions of gelatin chloride and gelatin phosphate are alike, with the exception of part of the descending

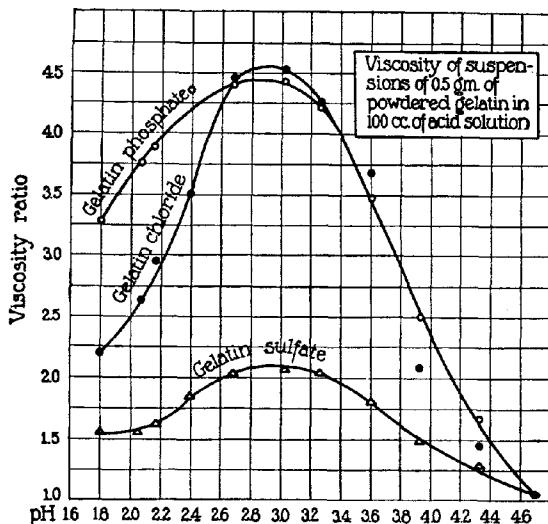


FIG. 79.—Viscosity of suspensions of 0.5 gm. of powdered gelatin of grain size 100 to 120. Abscissæ are the pH, the ordinates are the ratio of time of outflow of suspension to time of outflow of water. The influence of HCl and H_3PO_4 is practically identical for the same pH, while H_2SO_4 depresses the viscosity of the suspensions to a little less than one-half of that for HCl.

branch. This difference is probably due to incomplete electrolytic dissociation of the phosphoric acid.

Experiments on the influence of these three acids on swelling (Fig. 32, Chap. VII) show that the curves for the relative volume of powdered gelatin in solutions of these three acids are similar to the viscosity curves in Fig. 79, since the relative volume of gelatin sulphate was found to be not far from one-half of that of gelatin chloride or gelatin phosphate of the same pH.

We have seen that the viscosity of a gelatin chloride solution, *e.g.*, of pH 3.0, is lowered when neutral salts are added and the pH kept constant (Fig. 52, Chap. VIII). The same is true for the viscosity of suspensions of powdered gelatin. Doses of 0.5 gm. of powdered gelatin of pH 6.0, going through sieve 100 but not through sieve 120, were put each into 100 c.c. of water containing 6 c.c. of 0.1 *N* HCl, and different quantities of NaNO₃, so

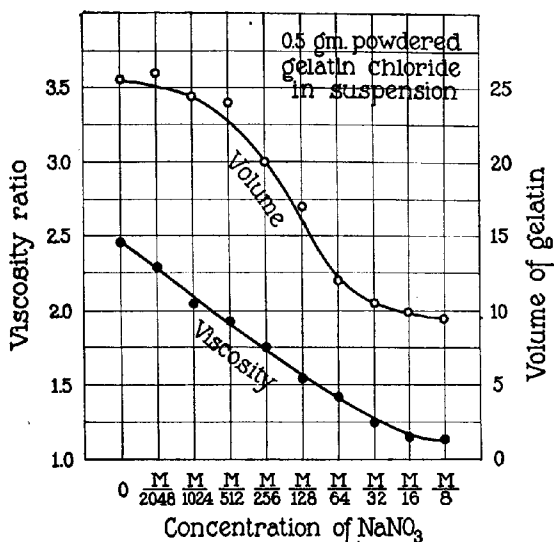


FIG. 80.—Showing depressing influence of neutral salts on viscosity of suspensions of powdered gelatin in water and on the volume occupied by the gelatin particles in the suspension.

that the concentration of the salt varied in the different solutions from $M/8$ to $M/2,048$. One solution contained no salt. The pH of the gelatin varied in the neighborhood of 3.0; the temperature was 20°C. After 2½ hours, when the Donnan equilibrium between the particles and the surrounding solution was supposed to be established, the viscosity of each suspension was measured at 20°C. and the volume occupied by the suspended particles of gelatin was ascertained in the manner described. It was found

that the addition of salt diminished the relative volume of the gelatin particles and the viscosity (Fig. 80). Where the volume of the gelatin was great it no longer varied parallel with the viscosity, as was to be expected from the fact that Einstein's formula no longer holds in this case.

The measurements of the pH of the gelatin solution and the outside solution showed that the addition of salt diminished the difference between the two, as Donnan's theory demands (Table XLIX).

TABLE XLIX

	Concentration of NaNO ₃							
	0	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32
pH of gelatin particles.....	3.04	3.04	3.03	3.02	3.00	3.02	2.97	2.94
pH of supernatant liquid....	2.74	2.76	2.76	2.76	2.77	2.80	2.78	2.77
Difference, pH inside minus pH outside.....	0.30	0.28	0.27	0.26	0.23	0.22	0.19	0.15

This demonstration completes the proof that the viscosity of *suspensions* of powdered gelatin in water of different pH is influenced in the same way by electrolytes as is the viscosity of *solutions* of the same gelatin salts, and that this influence is due, in the case of suspensions, to the influence of the Donnan equilibrium upon the swelling of the particles.

The volume V of gelatin occupied in 100 c.c. of the suspension was determined by filtering and deducting the volume of the filtrate from the total volume of the suspension. Knowing the viscosity, we can calculate Einstein's constant c according to the formula

$$\left(\frac{\eta}{\eta_0} - 1\right) \frac{100}{V} = c.$$

c should be 2.5 if V is sufficiently small.

The values in Table L show that Einstein's formula gives correct values for viscosity when the volume of the gelatin

TABLE I.

V, cubic centimeters	$\frac{\eta}{\eta_0}$	c
12.0	1.292	2.5
17.0	1.480	2.8
18.0	1.792	4.4
20.5	2.064	5.1
20.5	2.020	4.9
20.0	1.855	4.2
18.0	1.625	3.5
16.5	1.542	3.3

is small, since in that case c is equal, or nearly equal, to 2.5, as his formula demands.

When, however, the volume is larger, the value for c exceeds 2.5. The fact that the value for c exceeds 2.5 when the relative volume occupied by the particles in the solution is large was found also by Hatschek, Smoluchowski, and Arrhenius. We have stated already that Hatschek replaced the value 2.5 in Einstein's formula by a larger one, namely, 4.5. This, however, meets in our case with the difficulty that the value c shows a drift reaching a maximum when the volume of the gelatin particles is a maximum. This difficulty is largely avoided in Arrhenius's formula and we have to change from Einstein's formula to that of Arrhenius whenever the relative volume of the particles in solution or suspension exceeds the limits of the applicability of Einstein's formula, as we shall see in the next chapter.

The experiments on the viscosity of suspensions of powdered gelatin in water have, therefore, led to the result, first, that the influence of pH, of the valency of ions, and of the concentration of neutral salts on the viscosity of *suspensions* of finely powdered gelatin in water is similar to the influence of these three agencies on the viscosity of gelatin *solutions*; second, that the influence of electrolytes on the viscosity of the suspensions is due to the variation of the swelling (or relative volume) of the suspended particles; and third, that this latter fact explains why the Donnan equilibrium determines also the variation of viscosity of these suspensions.

CHAPTER XV

VISCOSITY (*Continued*)

1. The experiments in the preceding chapter dealt with suspensions of powdered gelatin in water. The question now arises as to whether the high viscosity of gelatin solutions is also due to the swelling of solid aggregates of gelatin suspended in the gelatin solution. If this is true, the viscosity of a gelatin solution should increase upon standing at a sufficiently low temperature, since on standing more and more aggregates are formed from the isolated molecules. That there exist submicroscopic solid aggregates, which may be considered the precursors of the continuous gel to which a gelatin solution sets on standing under the proper conditions, is not a mere assumption, but is proved by the observations of Menz.¹ This author also observed that the number and size of these particles increase on standing.

When a 0.5 per cent solution of isoelectric gelatin is heated rapidly to 45°C., cooled rapidly to a lower temperature, *e.g.*, 20°C., and kept at this temperature, the solution will ultimately set to a continuous gel but will steadily increase its viscosity before this stage is reached. It is natural to assume that the formation of a continuous jelly is preceded by the formation of submicroscopic pieces of jelly, which increase in number and size, forming finally a continuous jelly. Hence, the longer a solution of isoelectric gelatin stands at 20°C. the greater the number of submicroscopic solid pieces of jelly formed in the solution. The submicroscopic pieces of jelly surrounded by a true solution of isolated molecules of gelatin in water are compelled to regulate the amount of water they occlude by the Donnan equilibrium. Hence, when we add some HCl to a 0.5 per cent solution of isoelectric gelatin after the solution has been standing for some hours at 20°C., we should expect to find a higher viscosity than when we add the same amount of acid to the gelatin solution

¹ MENZ, W., *Z. physik. Chem.*, vol. 66, p. 129, 1909.

immediately after it has been rapidly heated to 45°C. and rapidly cooled to 20°C.

This experiment turns out as expected, as is shown in Fig. 81. When a 0.5 per cent solution of isoelectric gelatin is rapidly heated to 45°C., cooled rapidly to 20°C., and brought immediately to a different pH by the addition of HCl, at 20°C. a viscosity curve like the lowest in Fig. 81 is obtained. When, however,

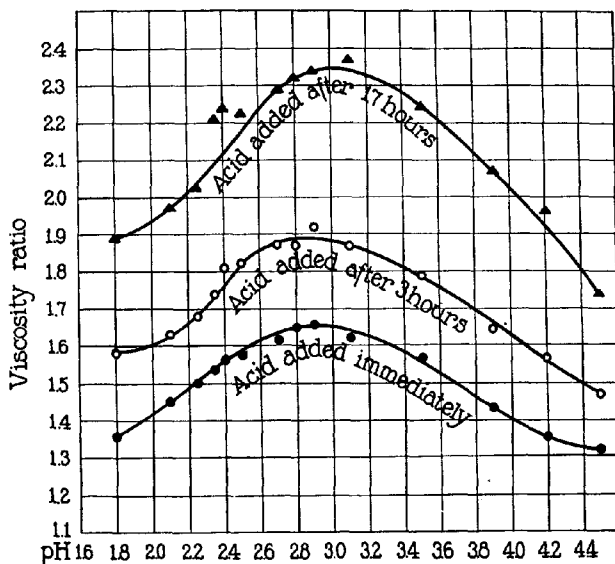


FIG. 81.—Increase in viscosity when acid is added to solutions of isoelectric gelatin after they had been standing for 3 and 17 hours, respectively.

the 0.5 per cent isoelectric gelatin solution is allowed to stand for 3 hours at 20°C. before the acid is added, a parallel viscosity curve is formed at 20°C. but higher than the first one (middle curve, Fig. 81), for the reason that during the 3 hours an additional number of solid jelly particles capable of swelling has been formed. If the solution of isoelectric gelatin stands for 17 hours at 20°C. before the HCl is added, the curve is still higher though practically parallel with the first curve (upper curve, Fig. 81), except at

the summit. It is probable that on standing not only the number but the size of individual particles increases, and the writer has observed that for the size of granules used in his experiments

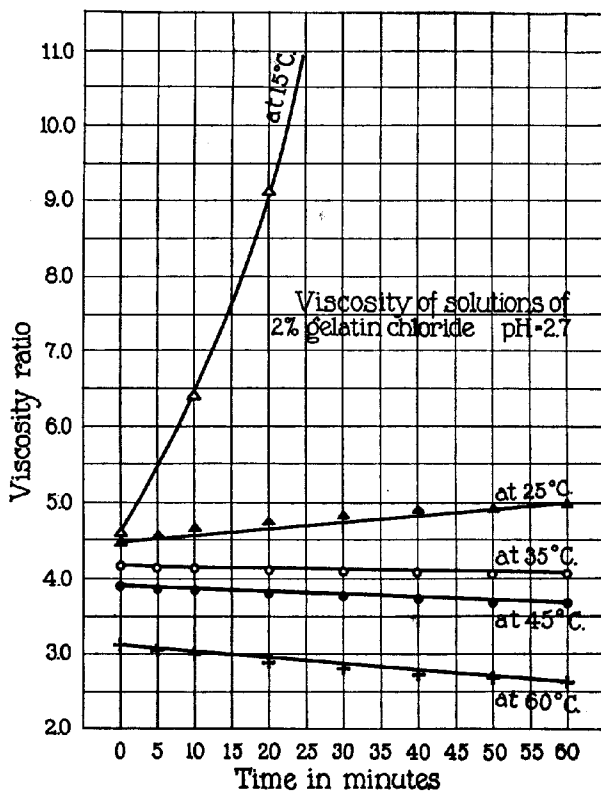


FIG. 82.—Influence of temperature on the variation of viscosity of gelatin solutions on standing. Below 35°C. the viscosity of a 2 per cent gelatin chloride solution of pH 2.7 no longer increases, but diminishes on standing.

the greater the size the greater the viscosity, since the viscosity is chiefly, but not exclusively, a function of the relative volume of the particles.

Since jelly formation of gelatin is a reversible process, we should expect that two opposite processes always take place simultaneously in a gelatin solution on standing, namely, first, the formation of solid particles of jelly through the aggregation of previously isolated gelatin molecules and ions as soon as their oily groups come into close contact, and second, the dissolution of such aggregates (micellæ) back into isolated molecules and ions, due to heat agitation. It is easy to show that powdered gelatin of a given pH dissolves the more rapidly the higher the temperature. If therefore our assumption is correct, that in a solution of gelatin two opposite processes go on constantly, the rate of melting of the micellæ should increase if the temperature rises. Hence, at very low temperature the viscosity of a gelatin solution should increase rapidly on standing, since the formation of new micellæ takes place constantly, while practically no melting of micellæ occurs. When, however, the temperature is raised beyond a certain point, the rate of melting of micellæ increases more rapidly than the rate of formation of new micellæ. Hence, at such a temperature the viscosity of a gelatin solution should not increase but decrease on standing.

This conclusion was tested experimentally and found to be correct. A 2 per cent solution of gelatin chloride of pH 2.7 was rapidly heated to a temperature of 45°C. and then rapidly brought to the temperature at which the change of viscosity of the solution with time was to be observed. At definite intervals the viscosity of the solutions was measured. Figure 82 gives the result. At 15°C. the viscosity increased rapidly on standing; at 25°C., it increased on standing, but less rapidly; at 35°C. or above it diminished on standing the more rapidly the higher the temperature. The temperature at which the two opposite processes—the formation and the melting of micellæ—occur equally rapidly in a 2 per cent solution of gelatin chloride of pH 2.7 lies near 35°C., according to Davis and Oakes¹ near 38°C.

When acid is added to powdered isoelectric gelatin the time required to dissolve the particles diminishes at a given temperature with increasing hydrogen ion concentration of the solution, and this tendency of the particles to dissolve with increasing hydrogen ion concentration shows no maximum as does the swell-

¹ DAVIS, C. E. and OAKES, E. T., *J. Am. Chem. Soc.*, vol. 44, p. 464, 1922.

ing. Hence we should expect that the more acid is added to a 0.5 per cent solution of isoelectric gelatin the less the viscosity will increase on standing at a given temperature, *e.g.*, 20°C., since the more acid is added to isoelectric gelatin the greater the

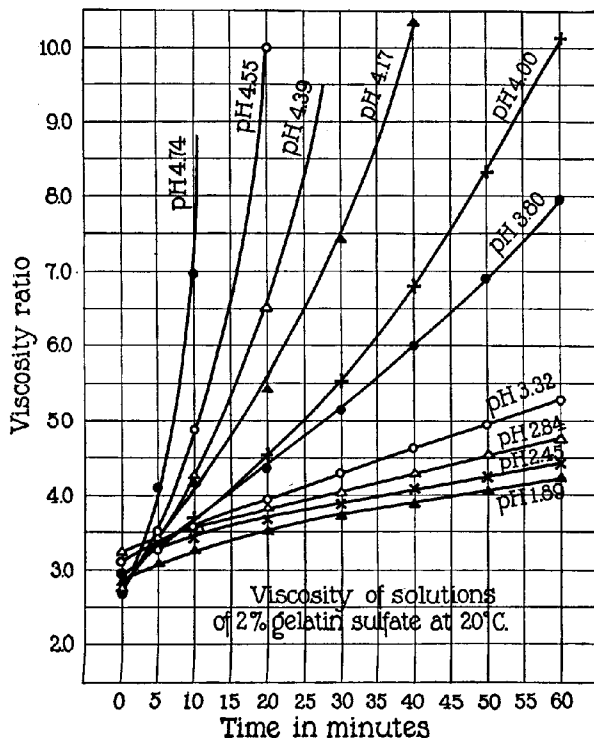


FIG. 83.—Increase of viscosity of gelatin sulphate solution of different pH on standing. The increase is most rapid at the isoelectric point, thus proving that the acid retards or prevents the formation of submicroscopic solid particles of jelly on standing.

tendency of the solid jelly particles already existing to dissolve; while the tendency of the isolated gelatin molecules or ions to adhere to each other is not increased. It should follow that on standing the viscosity of a 0.5 per cent solution of gelatin chlo-

ride or gelatin sulphate will increase the less at 20°C. the lower the pH of the solution. Figure 83 shows that this is the case.

We have shown in Part I that the rate of solution of powdered gelatin in water is influenced in a different way by different salts.

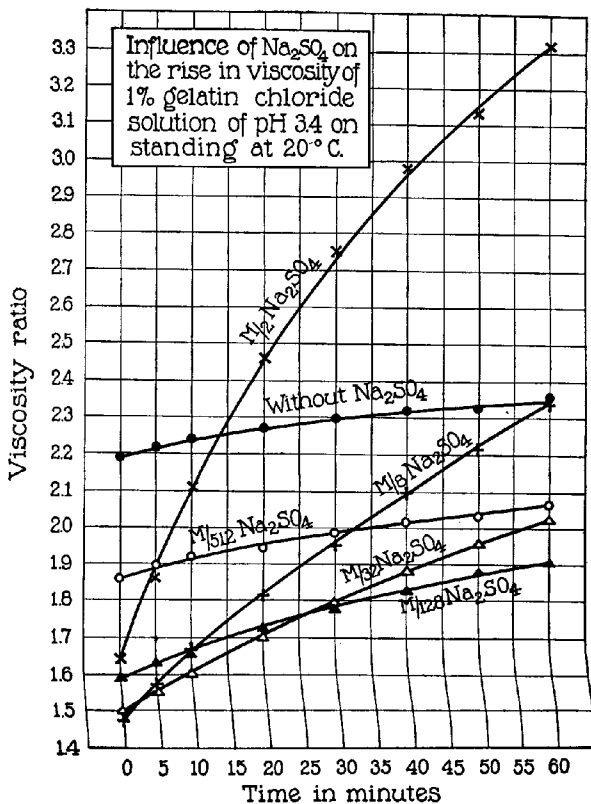


FIG. 84.—Showing that concentrations of Na_2SO_4 of $M/32$ and above cause an increase in the viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

Na_2SO_4 diminishes the rate of solution of powdered gelatin chloride when the concentration of Na_2SO_4 exceeds $M/64$; and the

diminution is the greater the higher the concentration; while CaCl_2 accelerates the rate of solution of powdered gelatin chloride when the concentration of CaCl_2 exceeds $M/4$.

Gelatin chloride solutions of pH 3.4, containing 1 gm. of originally isoelectric gelatin in a 100-c.c. solution, were made up in various concentrations of Na_2SO_4 and CaCl_2 . The solutions were rapidly heated to 45°C . and rapidly cooled to 20°C . and kept

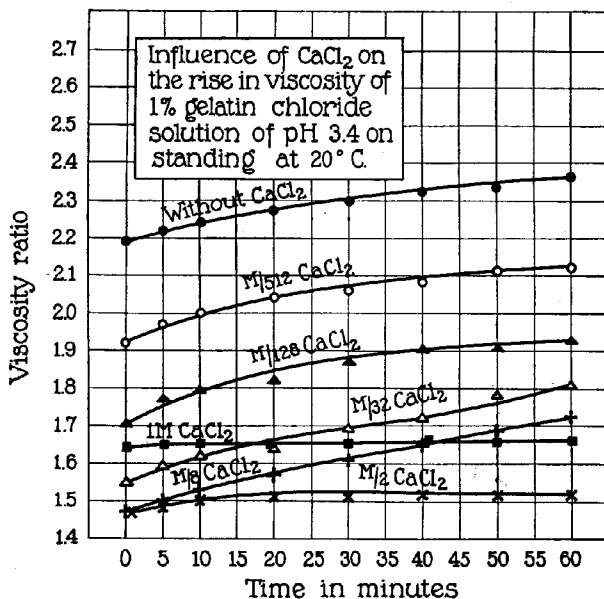


FIG. 85.—Showing that concentrations of CaCl_2 of $M/2$ or above prevent the increase in viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C .

at this temperature for 1 hour. The time of outflow of the solution through a viscometer was measured immediately and at intervals of 5 or 10 minutes. The time of outflow of water through the viscometer at 20°C . was 61 seconds.

The viscosity of a gelatin chloride solution of pH 3.4 rises very slowly (uppermost curve in Fig. 84) and the rate of increase of viscosity on standing is not materially altered in $M/512$ Na_2SO_4

and only little in $M/128$ Na_2SO_4 . In $M/32$ Na_2SO_4 the viscosity increases more rapidly on standing, in $M/8$ Na_2SO_4 still more rapidly, and in $M/2$ Na_2SO_4 very sharply. This is exactly what we should expect, since the Na_2SO_4 causes a diminution of the rate of solution of gelatin chloride as soon as the concentration of

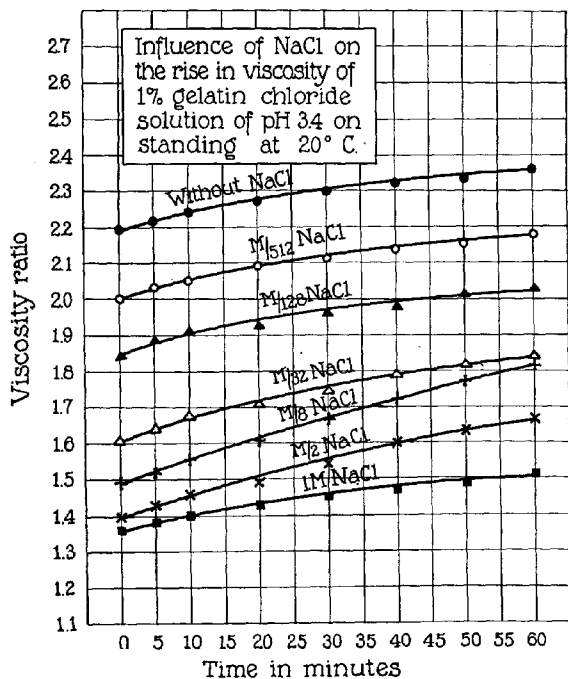


FIG. 86.—Showing that NaCl solutions up to a concentration of 1M have no effect on the increase in viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

Na_2SO_4 is above $M/64$. In such solutions the rate of solution of micellæ will be less and less, and since new micellæ are constantly formed at 20°C., the viscosity will rise more rapidly on standing when the solution contains Na_2SO_4 in concentrations above $M/64$ than when the solution contains less Na_2SO_4 or none at all.

Figure 85 shows that CaCl_2 in concentrations up to $\text{m}/8$ does not alter the increase in viscosity of gelatin chloride solution on standing, but that the viscosity of gelatin chloride of pH 3.4 no longer increases on standing when the concentration of CaCl_2 is $\text{m}/2$ or 1 m . In this concentration CaCl_2 causes a slight increase in the rate of solution of gelatin chloride.

NaCl causes no change in the rate of solution of gelatin chloride as long as the concentration of NaCl does not exceed 1 m . Above this concentration it causes coagulation and the viscosity can

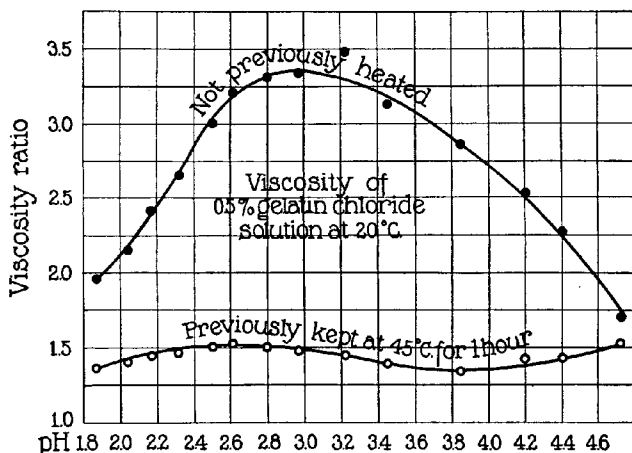


FIG. 87.—Showing that previous heating diminishes the viscosity of 0.5 per cent solutions of gelatin chloride.

no longer be measured. Hence NaCl in concentrations up to 1 m should not alter the rate of increase of viscosity of gelatin chloride solutions on standing. Figure 86 shows that this is correct.

The simplest method of melting solid particles of jelly is by heating to 45°C . If, therefore, the striking increase in viscosity which occurs when a 0.5 per cent solution of isoelectric gelatin is kept standing for a day at a temperature of, *e.g.*, 10°C ., is due to the formation of particles of solid jelly, then if this solution is heated to 45°C . and cooled rapidly to 20°C . the majority of these solid particles should have melted and dissolved into isolated ions

or molecules. Hence, such a solution when cooled rapidly to 20°C. should show at this temperature a considerably lower viscosity than the same solution shows at 20°C. when it is brought to this temperature directly from 10°C. without previous heating to 45°C. The experiment represented in Fig. 87 shows that this is the case.

These experiments, then, support the conclusion that the high viscosity of gelatin solutions and the influence of electrolytes on this viscosity are due to the fact that these solutions contain sub-microscopic particles of solid jelly (micellæ) capable of occluding large amounts of water the quantity of which is regulated by the Donnan equilibrium. In other words, the influence of electrolytes on viscosity is in the last analysis an influence on the swelling, *i.e.*, the osmotic pressure inside the particles due to the Donnan equilibrium.

2. The pH influences the viscosity of casein chloride solutions in a similar way to that in which it influences gelatin chloride solutions; and the depressing effect of neutral salts on the viscosity of casein chloride solutions is similar to that of the addition of salts on the osmotic pressure of gelatin chloride. Casein chloride solutions have no tendency to set to a jelly, but they have one feature in common with gelatin solutions, namely, the existence of particles capable of occluding water, the amount of which is regulated by the Donnan equilibrium. As a consequence, casein chloride solutions have a comparatively high viscosity, which is influenced by electrolytes in the way characteristic for the Donnan equilibrium. The existence of such particles in the casein chloride solution is indicated by the optical appearance of the solution.

The material used in our experiments was a fine dry powder of nearly isoelectric casein prepared after Van Slyke and Baker. Particles of equal size of grain (between mesh 100 and 120) were sifted out and 1 gm. of such powder was put into 100 c.c. each of solutions of HCl of different concentrations to bring the casein to varying pH. A microscopic examination of the granules showed that they underwent a swelling which was a minimum at the isoelectric point, which increased with increasing hydrogen ion concentration until it reached a maximum, and which then diminished again with a further increase in the hydrogen ion

concentration (see Chap. XIX). Hence, the volume of the casein particles suspended in the HCl varied in a similar way with the pH as the volume of suspended particles of gelatin.

This swelling could also be observed when the suspension was put into 100-c.c. graduates and the suspended particles were allowed to settle. The volume of the sediment was a minimum at the isoelectric point, increasing with increasing hydrogen ion concentration of the solution and finally decreasing again. But the curves of swelling and of volume of sediment were only

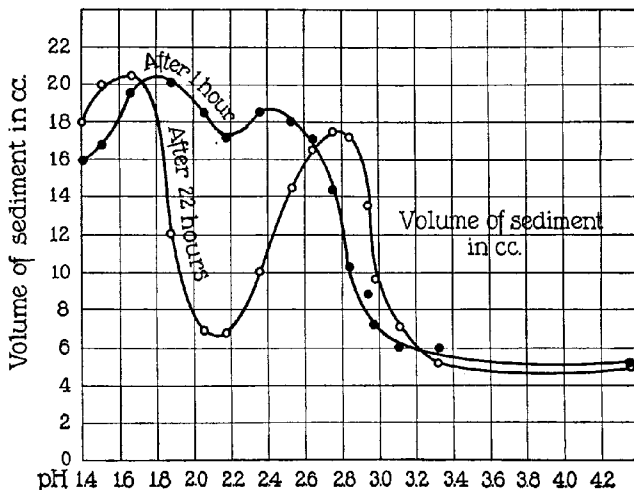


FIG. 88.—Swelling and solution of casein chloride in 1 and 22 hours at 20°C.

parallel at the beginning of the experiment, since the swelling (which occurred at once) was followed by some of the casein going into solution or into suspension. The longer the experiment lasted the smaller the volume of the sediment became and the larger the mass which went into the supernatant solution. This is expressed in Fig. 88. The upper curve represents the volume of the sediment after 1 hour. The suspension of 1 gm. of casein in 100 c.c. of HCl of different concentration was kept for 1 hour at 20°C., was shaken repeatedly but not frequently, and was then passed into 100-c.c. graduates and allowed to settle

at 20°C. After 2 hours the volume of the sediment was measured and the volumes are the ordinates of the curve marked "After 1 hour" in Fig. 88. A similar experiment was made in which the suspension of casein was kept for 22 hours at 20°C. and was allowed to settle during 6 hours also at 20°C. The volumes are the ordinates of the second curve in Fig. 88 marked "After 22 hours." The abscissæ are the pH of the total solution and suspension.

The curve "After 1 hour" is clear, since it is chiefly the expression of the variation of the degree of swelling of the casein

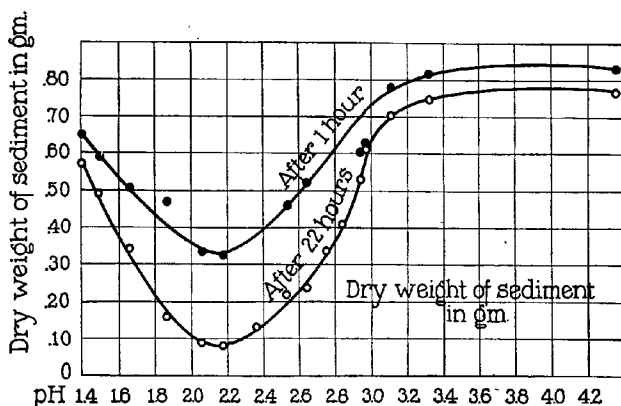


FIG. 89.—Dry weight of sediment of casein chloride solutions after 1 and 22 hours.

particles, not as much having gone into solution as after 22 hours. We notice that the volume occupied by the solid particles in the 1-hour curve is a minimum at the isoelectric point, that it rises steeply after pH 3.1, that it drops at 2.2, and that a second drop commences at pH 1.8. The two drops have a different cause. The drop at pH 1.8 is due to a diminution of the degree of swelling of the sediment, while the drop at 2.2 in the 1-hour curve is due to the fact that at pH 2.2, where the solubility of casein chloride is a maximum, some of the casein chloride has gone into solution. This conclusion is supported by the fact that the drop at 2.2 increases in time and is very considerable after 22

hours (see Fig. 88), while otherwise the 1-hour and the 22-hour curves show only minor differences.

The proof that this interpretation in the volume curves of Fig. 88 is correct is furnished by Fig. 89, where the ordinates are the dry weights of the sediments, the volumes of which are given in Fig. 88. One gram of powdered casein had, when dried for 24 hours at between 90 and 100°C., a dry weight of 0.87 gm.

That part of the casein chloride which goes into the supernatant liquid (*i.e.*, which is not contained in the sediment) consists of two constituents, namely, first, solid submicroscopic particles in suspension, which in due time would have settled, and second, isolated casein ions and molecules. The solid particles in the supernatant liquid (unless they are below the limit required to occlude water) undergo the same swelling under the influence of the Donnan equilibrium as the particles of the sediment. In addition, however, we have individual casein ions in solution (the molecules being probably insoluble, since isoelectric casein is practically insoluble), but these ions cannot undergo any swelling and hence do not add materially to the volume and the viscosity. As a consequence, the more solid particles of casein chloride are dissolved into isolated casein ions or particles too small to occlude water the more the relative volume occupied by the casein in the solution should be diminished, and this should be accompanied by a diminution in viscosity. If our theory of the origin of the viscosity of the gelatin solutions is correct, it should be possible to prove that where the solubility of the casein chloride solution is a maximum the viscosity curve shows a drop.

The correctness of this inference is supported by the viscosity curves in Fig. 90, which represent the viscosity after 1 hour and after 22 hours. The experiments are the same as those referred to in Figs. 88 and 89. The viscosity of the total suspension and solution was measured in a straight viscometer with a time of outflow for water of 48.4 seconds at 20°C. The curve for the viscosities after 1 hour is the expression chiefly of the swelling, since casein chloride goes only slowly into solution at 20°C. The curve is almost continuous and has its maximum in the region between pH 2.1 and 2.4, where also the swelling is a maximum. There is, however, a slight depression at pH 2.2, where the solubility of the casein is a maximum.

The curve for the viscosities (Fig. 90) after 22 hours shows, however, a distinct saddle at pH 2.2, where the solubility of casein chloride is a maximum. This agrees with the assumption that the high viscosity is due to swollen particles of casein, a certain quantity of which had been dissolved at or near pH 2.2. This solution of the particles capable of swelling beneath that size where they no longer can occlude water must diminish the relative volume of the casein and cause a diminution of the viscosity. Below a pH of 1.8, where the solubility of the casein is considerably diminished, the 1-hour and the 22-hour viscosity curves

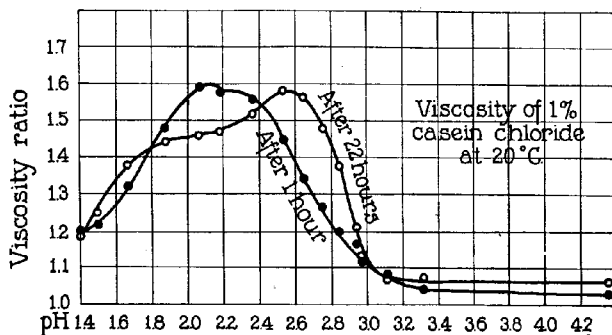


Fig. 90.—Viscosity of 1 per cent casein chloride solutions after 1 and 22 hours at 20°C.

(Fig. 90) no longer differ materially. As a consequence of the saddle, the maximum of the viscosity curve after 22 hours now lies at pH 2.6.

Since the point at issue, namely, the diminution of the viscosity when solid submicroscopic particles, capable of swelling, are dissolved into particles so small that they no longer can occlude water, is so fundamental for the theories of viscosity and of colloidal behavior in general, it seemed necessary to look for a more striking proof than that given in the experiment quoted. For this purpose measurements were made on 1 per cent casein chloride solutions prepared from very finely powdered casein particles sifted through a 200-mesh sieve. In order to get a more rapid solution of the particles the experiment was carried

out at 40°C. The time of outflow of water through the viscometer at 40°C. was 35.5 seconds. Figure 91 gives the results. The viscosity measurements were made at four different times, namely, immediately after the powdered casein was put into the HCl and after 1½, 3, and 6 hours. During this time the casein chloride solutions were kept at 40°C. The viscosity curve taken immediately after the suspensions were prepared is continuous and is the expression of the swelling which occurred in the few minutes which elapsed in the preparation of the suspensions and during which the casein was at 40°C. The maximum swelling

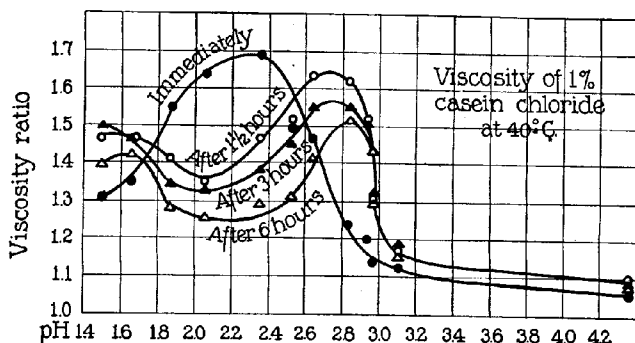


FIG. 91.—Diminution of viscosity through solution of solid particles of casein chloride.

occurred at about pH 2.3. At this time the amount of casein dissolved into separate casein ions was negligible. The curve resembles the 1-hour curve in Fig. 90. After 1½ hours the second measurements of viscosity were taken, and the reader will notice from Fig. 91 that the viscosity has dropped considerably in the neighborhood of pH 2.2, where the solubility of casein chloride is the greatest, the maximum depression being at pH 2.1, where also the solubility is a maximum. With a further lowering of the pH the viscosity rises again. The maximal viscosity in the 1½-hour series is now at pH of about 2.7 or 2.8 where it was also in the 22-hour series in Fig. 90. The later viscosity measurements, after 3 and 6 hours (Fig. 91), confirm these conclusions.

3. It is of interest to see whether or not Arrhenius's formula can account for the influence of electrolytes on the viscosity of casein suspensions. If this were the case, the curves representing $\log \frac{\eta}{\eta_0}$ should run parallel to curves representing the relative volume occupied by the casein in the solution. We get the values of $\log \frac{\eta}{\eta_0}$ from our observations of the relative viscosity which give us $\frac{\eta}{\eta_0}$, and we can calculate the volume from the

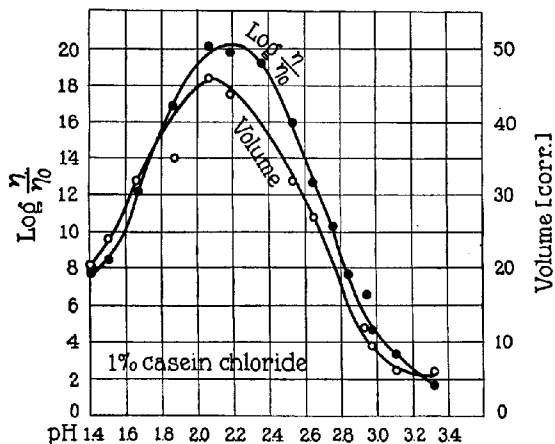


FIG. 92.—Similarity of curves for $\log \frac{\eta}{\eta_0}$ and for relative volume of casein chloride in solutions.

measured volume of the sediment plus the calculated volume of the casein in the supernatant liquid. The latter value is obtained by deducting the dry weight of the sediment from the (known) dry weight of the whole mass of casein put into the water (1 gm. powdered casein, dry weight = 0.87 gm.), assuming that the casein in the supernatant liquid consists exclusively of suspended particles. This is partly correct for a 1-hour experiment at 20°C. The ordinates in Fig. 92 represent the values for volume thus

corrected and the values for $\log \frac{\eta}{\eta_0}$, while the abscissæ are the pH of the suspensions. The two curves are almost parallel.

It should be stated that these corrected volumes of casein include a certain amount of water between the granules. We are, however, in this case not concerned with the absolute but only with the relative volume occupied by the casein.

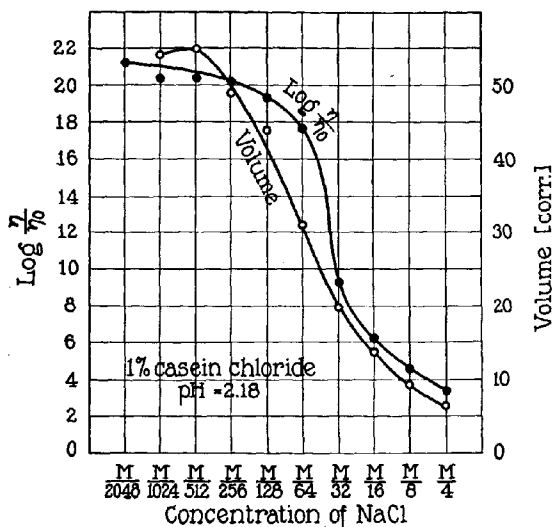


FIG. 93.—Similarity of curves for $\log \frac{\eta}{\eta_0}$ and for relative volume of casein chloride in solutions.

When NaCl is added in different concentrations to a casein chloride solution, it is noticed that the viscosity is diminished, as it is in the case of solutions of gelatin chloride. We shall see in Chap. XIX that this diminution of viscosity is accompanied by a diminution in the degree of swelling of the individual particles of casein which is parallel to the depression of the viscosity.

One gram of powdered casein was put into 100 c.c. of H_2O containing 12.5 c.c. of 0.1 N HCl, and NaCl in concentrations

varying from 0 to $M/4$. The mixture was shaken occasionally and kept for 16 hours at 20°C . Then the viscosity, volume of sediment (after settling for 24 hours), and dry weight of sediment (after deduction of the free NaCl contained in the sediment) were determined. When the volume and the values for $\log \frac{\eta}{\eta_0}$ are plotted as ordinates over the concentrations as abscissæ,

it is found that the two curves agree fairly well (Fig. 93), except where no or little salt was added and where, therefore, some casein particles had been completely dissolved. In this solution the calculated volume was too high and our curves express the fact. From these experiments we may conclude that the influence of electrolytes on the viscosity of casein solutions or suspensions is due to the swelling of particles of casein suspended in the solution of casein and that the volume of these particles is regulated by the Donnan equilibrium.

4. These experiments leave little doubt that the high viscosity of certain protein solutions, such as gelatin or casein, is due to the existence of solid particles occluding large quantities of water, the amount of which is regulated by the Donnan equilibrium, while the isolated ions of proteins in solution or the particles too small to occlude water have no share in the causation of high viscosities.

The quantities of water which can be occluded in a solid jelly of gelatin are enormous. If we assume the molecular weight of gelatin to be of the order of magnitude of about 12,000, a solid gel of 1 per cent originally isoelectric gelatin contains over 60,000 molecules of water to 1 molecule of gelatin. It is out of the question that such masses of water could be held by the secondary valency forces of the gelatin and water molecules. Casein particles occlude much less water, and for this reason the viscosity of casein chloride solutions never becomes as high as that of gelatin solutions containing equal masses of protein per 100 c.c. of solution.

All the experiments described agree with the occlusion theory but not with the hydration theory. Thus the fact that the viscosity of a 0.5 per cent solution of isoelectric gelatin increases rapidly at a temperature of 20°C . or below cannot possibly be explained on the basis of the hydration theory, since isoelectric

gelatin is not ionized. It might be explained on the basis of another suggestion which attributes to the gelatin solution a similar structure to that possessed by the solid jelly of gelatin. This idea would lead us to the assumption that in addition to the source of viscosity due to the relative volume of the protein solution there exists a second type peculiar to protein solutions which has no connection with the volume.

Bearing in mind the possibility that protein solutions may contain a preformed molecular structure analogous to that of the jellies or coagula which they can form, we are strongly impelled towards the belief that the type of viscosity which solutions of proteins exhibit may in some manner owe its existence to this structure, and not to the type of internal friction which hinders molecular and ionic motion. Thus a net-like structure, such as a tennis net, will offer no hindrance to the passage through it of a quickly moving body which is smaller than its meshes, other than that which is due to the fact that the material which composes the net occupies a small fraction of the area which the body must traverse, but to any force which involves deformation of the structure, for instance, a force which seeks to drag it through a small tube, it will offer a very considerable resistance.¹

This theory becomes untenable in the case of suspensions of powdered gelatin and of casein chloride which have no tendency to set to a jelly. It fails, moreover, to account for the fact that the influence of pH on the viscosity resembles that on the osmotic pressure of gelatin solutions. The assumption of a second type of viscosity independent of the relative volume occupied by the solute becomes unnecessary, since the theories of Einstein and of Arrhenius, respectively, which derive the viscosity from the relative volume, suffice to account for all the phenomena observed.

We therefore arrive at the conclusion that where the hydrogen ion concentration, the valency of ions, and the concentration of salts influence the viscosity of protein solutions in a similar way to that in which they influence the osmotic pressure, this influence on viscosity is in reality an influence of electrolytes on the swelling of solid submicroscopic protein particles contained in the solution.

¹ ROBERTSON, T. B., "The Physical Chemistry of Proteins," pp. 324-25, New York, London, Bombay, Calcutta, and Madras, 1918.

CHAPTER XVI

THE DIFFERENCE IN THE INFLUENCE OF AGGREGATES OF GELATIN ON OSMOTIC PRESSURE, VISCOSITY, AND MEMBRANE POTENTIALS¹

1. Genuine proteins form true solutions which may or may not contain, in addition to the isolated ions and molecules, submicroscopic particles capable of occluding water and of swelling. Only when a protein solution contains particles of this latter type do we notice a comparatively high viscosity and a similar influence of electrolytes on viscosity as on osmotic pressure. Solutions of crystalline egg albumin of not too high a concentration have a comparatively low viscosity which is not affected in the typical way by electrolytes, and this leads to the conclusion that these solutions consist chiefly of isolated ions and molecules or of particles too small to occlude water. If this conclusion is justified, we are forced to the further conclusion that the influence of electrolytes on the osmotic pressure of protein solutions is determined by the isolated ions of a protein solution and not by the submicroscopic particles capable of occluding water, since solutions of crystalline egg albumin show the influence of electrolytes on their osmotic pressure in a striking way. It would further follow that in case of a gelatin solution, where both isolated ions and submicroscopic micellæ are supposed to exist, the isolated ions are responsible for the influence of electrolytes on the osmotic pressure of the solution, while the submicroscopic particles of solid jelly capable of occluding water are responsible for the influence of electrolytes on the viscosity of gelatin solutions. In other words, the transformation of the submicroscopic particles of solid jelly into isolated molecules and ions should lower the viscosity and raise the osmotic pressure of a gelatin solution, and *vice versa*. It can be shown that this conclusion is supported by observations on gelatin solutions.

¹ LOEB, J., *J. Gen. Physiol.*, vol. 4, pp. 97, 769, 1921-22.

It was noticed in the preceding chapter that the viscosity of solutions of gelatin chloride does not always increase on standing, but that it diminishes when the temperature exceeds a certain limit. This was shown for a 2 per cent solution of gelatin chloride of pH 2.7 in Fig. 82. The viscosity of such a solution increases very rapidly on standing at 15°C., much less rapidly at 25°C., but diminishes when kept at a temperature above 35°C., and the more rapidly the higher the temperature. This we assume to be due to the fact that at a temperature above 35°C. the rate of

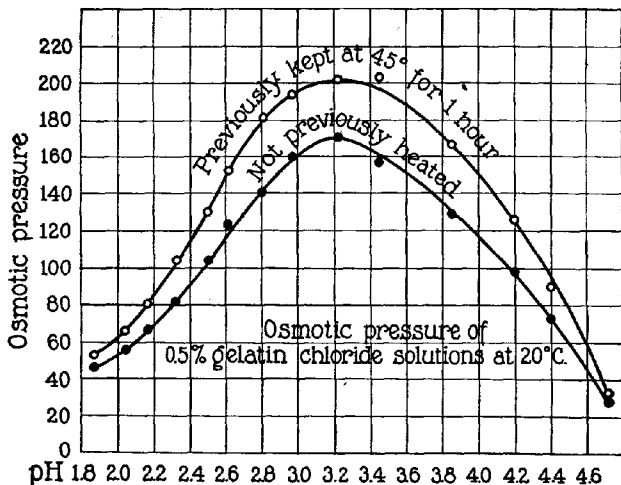


FIG. 94.—Showing that the osmotic pressure of a solution of gelatin chloride which has been previously heated to 45°C. for 1 hour and then rapidly cooled to 20°C. is higher than the osmotic pressure of the same solution of gelatin chloride not previously heated.

melting of submicroscopic particles of solid jelly exceeds the rate of their formation from isolated ions or molecules.

Several liters of a 0.55 per cent solution of isoelectric gelatin were kept at about 10°C. for 48 hours and at 20°C. for the next 24 hours. Then the stock solution was divided into two parts. One part was subdivided into doses of 90 c.c. each, and each was brought to a different pH by adding 10 c.c. containing different quantities of HCl. In this way the concentration of

originally isoelectric gelatin was, therefore, in every case 0.5 per cent. The second portion was treated in the same way, except that before adding the acid the gelatin was kept for 1 hour at 45°C. This was done to melt part of the submicroscopic pieces of jelly assumed to exist in the solution, and thus to increase the concentration of the isolated ions and molecules and to diminish the relative quantity of solid submicroscopic particles responsible for the high viscosity characteristic of gelatin solutions. After this second portion of the stock solution of isoelectric gelatin had been kept for 1 hour at 45°C., it was rapidly cooled to 20°C., the HCl was added in the way described for the first portion, and the solutions were put into collodion bags to measure the osmotic pressure. Each collodion bag contained about 50 c.c. of gelatin solution. The temperature now remained constant at 20°C. for both sets of experiments. It was noticeable from the beginning that the osmotic pressure of the gelatin solution which had been kept for 1 hour at 45°C., and which was therefore supposed to have melted into smaller particles, was higher than that of the gelatin solution not previously heated. Figure 94 shows the result after 22 hours. The maximum osmotic pressure was 200 mm. H₂O for the gelatin solution that had been previously heated, while it was only 170 mm. for the other gelatin solution not previously heated to 45°C.

Then the viscosities were determined at 20°C. and they gave the opposite result (Fig. 87 of the preceding chapter), the viscosities being considerably higher in the solutions not previously heated to 45°C. than in the solutions previously heated. This experiment then confirms our expectation that a transformation of solid submicroscopic particles of jelly into isolated protein ions and molecules diminishes the viscosity but increases the osmotic pressure of the solution.

As far as the quantitative relations are concerned, the difference in viscosity (Fig. 87) is more striking than the difference in osmotic pressure (Fig. 94). This is possibly connected with the fact that the lowering in viscosity due to heating to 45°C. was measured immediately after the temperature had reached 20°C. again, while the osmotic pressure of the same solutions was measured after the solutions had been standing for 22 hours at 20°C. During this time a considerable formation of submicro-

scopic particles of solid jelly had probably occurred in the solutions previously heated to 45°C.

It was expected that when we put a collodion bag filled with a 1 per cent solution of gelatin, *e.g.*, of pH 3.5, which had been kept for 1 hour at 45°C. and cooled to 20°C. into a beaker containing a 1 per cent solution of the identical gelatin chloride solution of pH 3.5, but which had not been heated to 45°C. before being brought to 20°C., water would diffuse from the latter into the former solution. This experiment was carried out with a positive result.

These experiments support the idea expressed in the preceding chapter that protein solutions are true solutions which may or may not contain solid particles of protein capable of swelling. In the case of gelatin solutions the formation of submicroscopic particles of solid jelly from isolated molecules or ions is a reversible process.

This probably explains a phenomenon which has puzzled the writer for a long time, namely, that the osmotic pressures of gelatin solutions of the same pH and concentration of originally isoelectric gelatin occasionally showed variations for which he could not account. It now becomes probable that this was due to a factor which was not taken into consideration, namely, that on standing at room temperature a gradual transformation of isolated molecules or ions into larger aggregates takes place, which must diminish the osmotic pressure but increase the viscosity. This source of variation was eliminated in the viscosity experiments in which the gelatin solution was always heated first to 45°C. and then as soon as this temperature was reached the solution was cooled to the temperature desired for the viscosity measurements. It is probable that the same uniformity of treatment is also required for the osmotic pressure experiments.

Solutions of Na caseinate are less opaque than those of casein chloride (of the same concentration of originally isoelectric casein), which indicates that the Na caseinate solution contains more isolated casein ions and fewer submicroscopic solid particles than the solution of casein chloride.

The writer has already shown in a preceding chapter that the maximal viscosity of a 1 per cent solution of casein chloride is higher than the viscosity of solutions of Na caseinate of equal concentration of originally isoelectric casein, while the osmotic

pressures of solutions of the two salts show exactly the reverse relation, the maximal osmotic pressure of a 1 per cent solution of Na caseinate being almost 700 mm. H_2O , while the maximal osmotic pressure of a 1 per cent solution of casein chloride is only about 200 mm.

The solutions of crystalline egg albumin seem to consist (at ordinary temperature and at not too high a concentration of albumin and of the hydrogen ions) exclusively or almost exclusively of isolated molecules or ions. Since the latter cannot diffuse through a collodion membrane, they give rise to a Donnan equilibrium across the membrane and hence only the osmotic pressure of solutions of salts of crystalline egg albumin is influenced by electrolytes in the way demanded, while the viscosity shows such an influence only to a negligible degree.

2. It should be possible to give a more striking confirmation of the relation between the viscosity and the osmotic pressure if we replace in a gelatin solution part of the dissolved gelatin by equal weight of powdered gelatin. Such a substitution should increase the viscosity and diminish the osmotic pressure of the solution.

Figure 95 shows that the osmotic pressure of a 1 per cent solution of originally isoelectric gelatin diminishes the more the more we replace the dissolved gelatin by small granules of powdered gelatin. The ordinates of the upper curve represent the values of the osmotic pressure of a 1 per cent solution of originally isoelectric gelatin at different pH, the pH serving as abscissæ of the curves. The acid used was HCl, and the curve is the usual one. At the beginning of the experiment the gelatin solution was rapidly heated to a temperature of 45°C. and rapidly cooled to 20°C. and then kept at that temperature throughout the entire experiment. The pH is that of the gelatin solution at the end of the experiment.

The middle curve represents an experiment in which 0.5 gm. of the isoelectric gelatin in solution was replaced by 0.5 gm. of isoelectric powdered gelatin. The latter did not contribute to the osmotic pressure, the observed osmotic pressure being due to the isolated ions of the 0.5 per cent gelatin in solution which determined the Donnan effect, and in addition to the osmotic pressure of the isolated gelatin ions and the isolated gelatin mole-

cules. Theoretically, of course, the coarse particles of gelatin also participate in the osmotic pressure, but this effect is negligible on account of the small number of such particles. (The gelatin particles used were of grain size slightly above 0.042 cm. diameter). At the beginning of the experiment the 0.5 per cent

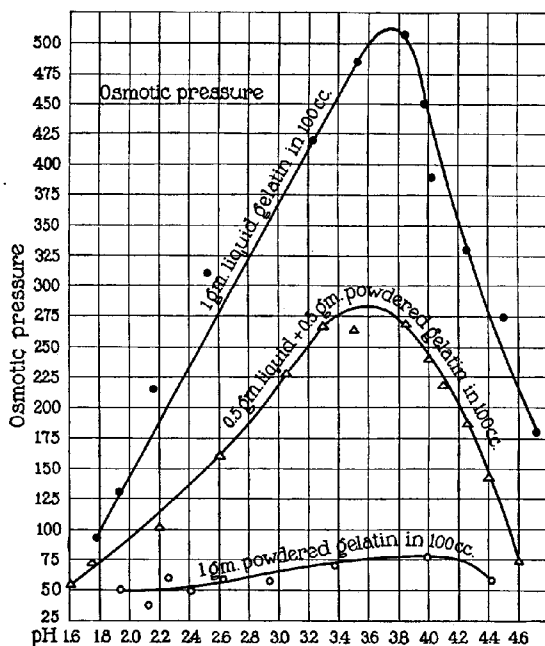


FIG. 95.—A suspension of 1 gm. of a fine powder of gelatin in 100 c.c. of water has practically no osmotic pressure (lowest curve), while a solution of 1 gm. of the same gelatin has a maximal osmotic pressure of over 500 mm. (uppermost curve). A mixture of 0.5 gm. of powdered and 0.5 gm. of liquid gelatin in 100 c.c. of water has practically the osmotic pressure of the 0.5 per cent liquid gelatin in 100 c.c. of water (middle curve).

solution of gelatin was rapidly heated to 45°C. and rapidly cooled to 20°C., and then the powdered gelatin was added. The pH is that of the 0.5 per cent gelatin solution at the end of the experiment.

The lowest curve represents the osmotic pressure of 1 gm. of powdered isoelectric particles in 100 c.c. of HCl of different pH. The slight osmotic pressure observed is probably due to the fact that a little of the gelatin went gradually into solution. This apparently happened to a less extent in a repetition of this experiment and the osmotic pressures observed were still lower than in the lowest curve in Fig. 95. All these osmotic pressure experiments were made in a thermostat at 20°C.

The viscosity is affected in exactly the opposite sense from the osmotic pressure if part of the dissolved gelatin is replaced by solid particles of gelatin. The more dissolved gelatin is replaced by solid particles of gelatin the higher the viscosity, a result to be expected from the experiments and conclusions already stated.

Solutions of 0.5, 0.625, 0.750, 0.875, and 1.0 gm. of isoelectric gelatin were heated quickly to 45°C. and cooled quickly to 20°C., and so much powdered gelatin of pH 7.0 was added as to bring the total gelatin in 100 c.c. to 1 gm.; *i.e.*, to a 0.5 per cent solution of gelatin was added 0.5 gm. of powdered gelatin (between mesh sizes 100 and 120), and to a 0.875 per cent solution of liquid gelatin was added 0.125 gm. of powdered gelatin, while no powdered gelatin was added to the 1 per cent solution of liquid gelatin. The different mixtures were brought to different pH through the addition of different quantities of HCl and the solutions were allowed to stand for 1 hour before the viscosities were measured in order to give the powdered gelatin a chance to swell. The results of the measurements are represented in Fig. 96. The reader will see that within the range of pH 3.6 and 1.4 the viscosity is the greater the more liquid gelatin is replaced by powdered gelatin. This supports the idea that the influence of electrolytes on the viscosity of gelatin solutions is due to the influence of the electrolytes on the swelling of solid submicroscopic particles of gel in the solution.

The nature of the curves in Fig. 96 between pH 4.6 and 3.8 requires an explanation. The curves are here the lower the more liquid gelatin is replaced by solid gelatin, because it was necessary to let the suspensions stand for at least 1 hour to allow the particles of powdered gelatin to swell before the viscosity measurements were made, and during this time the liquid

gelatin at or near the isoelectric point increases rapidly in viscosity, while this increase in viscosity is suppressed where the hydrogen ion concentration is higher. This is proved by Fig. 97, which gives the viscosity of the supernatant solutions of gelatin (without the suspended particles) which had been standing for 1 hour. Inside the range of pH 4.4 and 4.6 the viscosity had

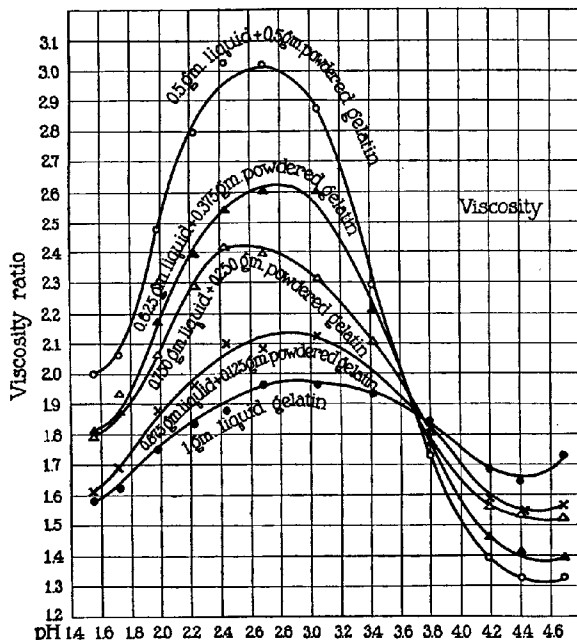


FIG. 96.—The influence of replacing liquid by powdered gelatin on viscosity is exactly the reverse as on osmotic pressure. The more the powdered particles replace the liquid gelatin the higher the viscosity.

risen more rapidly on standing than at the lower pH, which means that at or near the isoelectric point new submicroscopic particles of solid jelly are constantly formed from the molecules, while this process is the slower the higher the hydrogen ion concentration. While thus the addition of acid to a solution of isoelectric gelatin retards the rate of formation of new submicro-

scopic particles of jelly, it increases the swelling of those already present when the acid is added. On the other hand, powdered particles of isoelectric gelatin in water of pH 4.7 do not increase their volume on standing.

The fact that the addition of acid to a solution of isoelectric gelatin inhibits or retards the formation of new solid particles on standing was discussed in the preceding chapter.

If we now return to the discussion of the curves in Fig. 96, we may say that the results in those parts of the curves which belong to the abscissæ of pH above 3.8 are the expression of the fact that

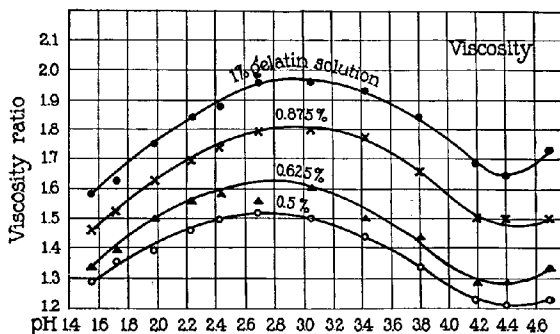


FIG. 97.—Viscosity of gelatin solutions after standing for 1 hour at 20°C. Notice minimum at pH 4.4, indicating that the viscosity has risen more near the isoelectric point on account of the formation of submicroscopic particles of gel.

that part of the viscosity which is due to the gelatin in solution had undergone an increase during the hour the solution had been standing at 20°C. after having been heated to 45°C.; and that the increase caused in the viscosity of the liquid gelatin was a maximum at the isoelectric point, being almost zero at a pH below 3.4, while the addition of acid had the opposite effect on the solid granules of gelatin, since their volume was increased according to the rules of the Donnan equilibrium.

A few remarks may be added concerning the influence of suspended particles of gelatin on the membrane potentials of a gelatin solution.

Powdered gelatin going through the meshes of sieve 30 but not through 60 was rendered isoelectric in the way previously de-

scribed. Part of this isoelectric gelatin was melted and the melted and the powdered isoelectric gelatin were mixed. The total weight of isoelectric gelatin in a 100-c.c. solution was always the same, but the proportion of powdered to dissolved gelatin varied, as indicated in Table LI. Thus, when the weight of the powdered gelatin was 0.5 gm., the weight of the dissolved gelatin was about 0.3 gm.; when the weight of the powdered gelatin was 0.2 gm., that of the dissolved was 0.6 gm., etc. One hundred cubic centimeters of the mixture contained 8 c.c. of 0.1 *N* HCl, and the pH of the gelatin solution (at the equilibrium condition to be described) was between 3.2 and 3.3. At this pH the osmotic pressure of a gelatin solution is nearly a maximum.

TABLE LI.—INFLUENCE OF SUBSTITUTION OF POWDERED FOR DISSOLVED GELATIN ON OSMOTIC PRESSURE AND MEMBRANE P.D.

Powdered gelatin per 100 c.c., grams.....	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.0
Dissolved gelatin per 100 c.c., grams.....	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Osmotic pressure.....	85	132	181	230	268	310	342	406	398
pH inside.....	3.16	3.20	3.18	3.19	3.22	3.27	3.28	3.30	3.33
pH outside.....	2.82	2.85	2.85	2.83	2.83	2.85	2.82	2.84	2.87
pH inside minus pH outside.....	0.34	0.35	0.33	0.36	0.39	0.42	0.46	0.46	0.46
Hydrogen electrode P.D. (millivolts).....	20.0	20.5	19.0	21.0	22.5	24.5	26.5	26.5	26.5
Membrane P.D. (millivolts).....	Between 23.0 and 18.0	Between 22.0 and 18.0	23.0	21.0	22.5	25.0	26.0	26.5	27.0
	No constant reading.								

Collodion bags of a content of about 50 c.c. were filled with these suspensions and closed with rubber stoppers perforated with glass tubes serving as manometers to measure the osmotic pressure. The bags were put overnight at 21°C. into beakers containing each 350 c.c. of 0.001 *N* HCl in water. The next day the osmotic pressure was read, the P.D. between the gelatin chloride solution and the outside aqueous solution free from gelatin was measured (with a Compton electrometer and indiffer-

ent saturated KCl-calomel electrodes), and the pH inside and outside was determined with the hydrogen electrode. Table LI gives the results of these observations.

The table confirms the observation that the osmotic pressure of the gelatin solution diminishes the more the more of the dissolved gelatin is replaced by powdered gelatin. The latter obviously does not participate in the osmotic pressure.

The table shows furthermore that the membrane P.D. observed at equilibrium between the gelatin solution and the outside aqueous solution varies much less than the osmotic pressure. It became necessary to ascertain whether or not this P.D. across the membrane which was measured with the aid of two indifferent electrodes (saturated KCl-calomel solution) was actually determined by the difference in the hydrogen ion concentrations inside and outside, as we should expect if the membrane potentials are due to a membrane equilibrium. The hydrogen electrode potentials were therefore measured. The reader will notice that the difference between the observed membrane potential (measured with indifferent electrodes) and the hydrogen electrode P.D. is not more than 0.5 millivolt. This leaves no doubt that the observed P.D. is determined by the difference in the hydrogen ion concentration on the opposite sides of the collodion membrane and that this P.D. obeys Donnan's equilibrium equation.

These facts show then that the protein aggregates participate in the Donnan equilibrium of a gelatin solution almost to the same extent as do the isolated molecules or ions of gelatin, and this participation finds expression in the fact that the membrane potentials are lowered comparatively little when dissolved gelatin is replaced by powdered gelatin. The same particles, however, do not contribute to the osmotic pressure, because their share in the excess of chlorine ions is contained inside the solid particles, where it serves to increase the swelling of the particles. In our experiment there exists inside of each particle of powdered gelatin a Donnan equilibrium whereby the concentration of Cl ions inside is greater than outside and this causes an osmotic pressure. Water will, therefore, diffuse into each granule until the cohesion pressure of the solid particles of gelatin equals the osmotic pressure inside the particles due to the Donnan equilibrium, and the particles will swell. When we therefore have a mixture of

dissolved gelatin and powdered particles (micellæ), we have two different osmotic pressures, namely, first, the osmotic pressure of the gelatin in true solution, and second, the osmotic pressure inside each solid particle of gelatin. The former is measured by the hydrostatic pressure of the column of water required to equalize the rate of diffusion in opposite directions through the membrane. This is the osmotic pressure of the protein solution in Table LI. The osmotic pressure inside each particle of solid powdered gelatin results in swelling, *i.e.*, in an increase of the force of cohesion between the molecules of the gel particle, and this effect does not appear in the osmotic pressure of the solution. Only that part of the osmotic forces in a protein solution appears in the form of hydrostatic pressure which is directly or indirectly due to the isolated molecules of the protein; and this hydrostatic pressure is diminished when part of the protein in solution is replaced by aggregates or micellæ of protein.

These facts make it therefore appear that the influence of electrolytes on the osmotic pressure of a solution of gelatin salt depends primarily on the isolated protein ions; that the similar influence of electrolytes on the viscosity of solutions of gelatin salts depends primarily upon the ionized aggregates in the solution; while the influence of electrolytes on the membrane potentials of solutions of gelatin salts depends on both isolated gelatin ions and ionized aggregates.

This difference in the influence of the degree of dispersion of ionized gelatin particles on the osmotic pressure and viscosity of solutions of gelatin salts makes it impossible to explain the similarity of the influence of electrolytes on the viscosity and osmotic pressure of gelatin solutions on the assumption that this similarity is due to the action of electrolytes on the degree of dispersion.

CHAPTER XVII

MEMBRANE POTENTIALS AND CATAPHORETIC POTENTIALS OF PROTEINS¹

1. Hardy observed in 1900 that particles of denatured (boiled) white of egg migrated in an electric field to the cathode in an acid solution and to the anode in alkaline solution, and did not migrate at all at a point between the two, namely, at the so-called isoelectric point.² It was shown in Chap. XI that the same influence of the pH on the charge of the protein exists in the case of membrane potentials, inasmuch as a protein solution enclosed in a collodion bag and submerged in water free from protein is positively charged on the acid side of the isoelectric point, negatively on the alkaline side, and not charged at all at the isoelectric point. The question arises: What is the cause of the similarity of the influence of the pH on the two types of potentials? The membrane potentials are due to a difference in the concentration of a diffusible ion, *e.g.*, the H ion inside the protein solution and outside, as is proved by the fact that the membrane potentials agree with the hydrogen electrode potentials of protein solutions. The cataphoretic potentials, however, are determined by the potential difference between the two strata of an electrical double layer situated at the interface between particles and water, but entirely in the water. One stratum or film of this double layer, namely, the one adjoining the solid particle, adheres to the solid particle and moves with it, and the charge of this film is the cause of the cataphoretic motion of the particles.

According to the theory of these double layers originally developed by Helmholtz and modified in an essential point by Perrin, the potential difference of this electrical double layer can be calculated from measurements of the velocity of migration of

¹ LOEB, J., *J. Gen. physiol.*, vol. 5, p. 505, 1922-23.

² HARDY, W. B., *Proc. Roy. Soc.*, vol. 66, p. 110, 1900.

such particles in an electric field with the aid of the following formula:

$$v = \frac{\epsilon \cdot E \cdot K}{4\pi\eta},$$

where v is the velocity of migration of the particle in centimeters per second, ϵ the potential difference between the two strata of the double layer around the solid particle, E the potential gradient in E.S.U. per centimeter of the galvanic field, K the dielectric constant of the water or the solution, and η the viscosity of the water. It may be said that this formula must be nearly correct because flocculation of suspensions of a given substance always occurs at the same calculated cataphoretic P.D., which would be impossible if the Helmholtz-Perrin formula were not, at least approximately, correct.

We are not so well informed as to the origin of the P.D. of this double layer, but we may assume with a good degree of probability that it is due to the fact that the two oppositely charged ions of an electrolyte are not contained in the same concentration in the two strata of the double layer, and that forces inherent in the water drive an excess of one type of ions—generally the OH or some other negative ion—into the outermost surface of the water, *i.e.*, into that film or stratum of the interface which adheres to and moves with the solid particle. Since this film determines the cataphoretic sign of charge of the particle, we notice that very frequently suspended particles are negatively charged in water, while the bulk of water, having a corresponding excess of positive ions, is positively charged.

It is obvious therefore that, as a rule, the cataphoretic potential has an entirely different origin from the membrane potentials, and this makes it more difficult to account for the fact that the sign of charge of membrane potentials and of cataphoretic potentials of protein particles varies in the same sense with the change in the hydrogen ion concentration.

It was therefore important to find out how far the agreement between the two potentials actually goes. For this purpose measurements of the influence of salts on the cataphoretic potentials of solid protein particles were made, using the microscopic method of Ellis¹ of measuring the velocity of migration in an

¹ ELLIS, R., *Z. physik. Chem.*, vol. 78, p. 321, 1911–12; vol. 80, p. 597, 1912.

electric field, and using Northrop's apparatus¹ with non-polarizable electrodes.

Such measurements were carried out on solid particles of four different proteins, casein, denatured egg albumin, collodion particles coated with gelatin, and collodion particles coated with genuine egg albumin.² The influence of salts on the cataphoretic potential was practically the same in the case of all these proteins and it may, therefore, suffice to confine ourselves here to a description of the results obtained with casein particles.

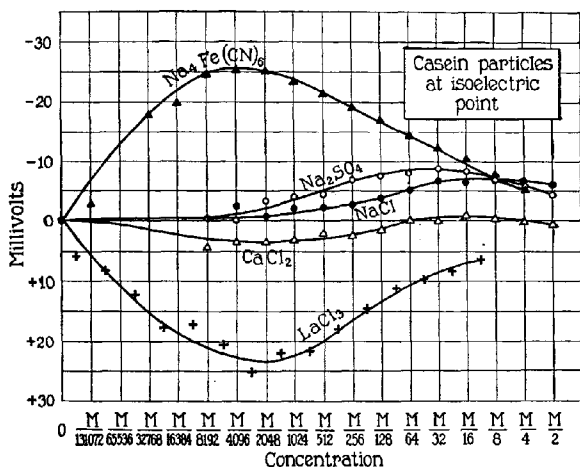


FIG. 98.—Influence of salts on the cataphoretic P.D. of casein particles at pH 4.7 (isoelectric point). Without salts the particles are not charged. In the presence of $\text{Na}_4\text{Fe}(\text{CN})_6$ they are negatively, and in LaCl_3 they are positively charged. In Na_2SO_4 the particles are slightly negatively, in CaCl_2 slightly positively charged.

The method of preparing the casein particles for these experiments was as follows: A 1 per cent solution of casein in HCl of pH of about 2.8 was titrated slowly with 0.1 N NaOH until the solution became very milky and almost ready to flocculate. The suspension was then centrifuged and the sediment obtained was ground in a mortar and made up to a creamy stock suspension

¹ NORTHROP, J. H., *J. Gen. Physiol.*, vol. 4, p. 629, 1921-22.

² LOEB, J., *J. Gen. Physiol.*, vol. 5, p. 395, 1922-23.

with a small amount of water to a pH of about 4.0. Four drops of such a stock suspension of casein particles were added to 50 c.c. of various concentrations of the salt solution of the desired pH. The particles remained in this latter solution for 20 minutes at room temperature and the rate of their migration in an electrical field was then measured in Northrop's apparatus.

Figure 98 gives the influence of the five salts on the cataphoretic P.D. of casein particles at the isoelectric point (pH 4.7). The

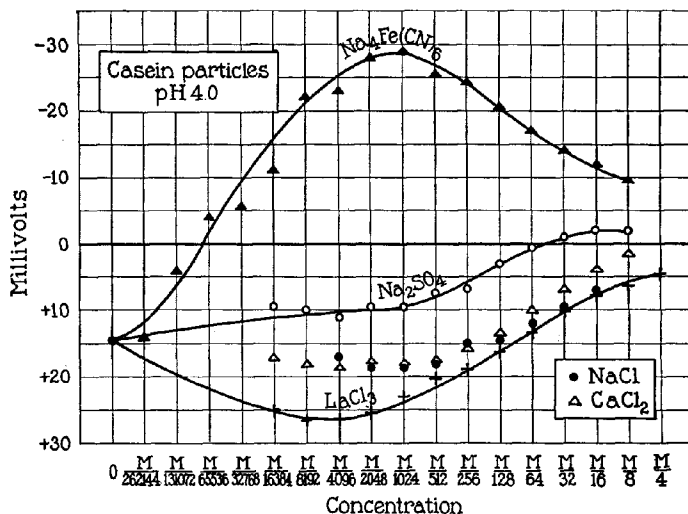


FIG. 99.—Influence of salts on the cataphoretic P.D. of casein particles at pH 4.0.

abscissæ are the concentrations of the salt, the ordinates are the P.D. of the double layer in millivolts. The ordinates are downward when the particles have a positive and upward when the particles have a negative charge. It is obvious that without salt the charge of the particles is zero. The addition of NaCl, Na₂SO₄, and CaCl₂ had very little effect on the cataphoretic P.D., except that CaCl₂ made the particles slightly positive and Na₂SO₄ slightly negative. While these effects were very minute, they seemed to exist in the case of all proteins thus far investigated.

The effects of LaCl_3 and of $\text{Na}_4\text{Fe}(\text{CN})_6$ on the P.D. of the isoelectric casein particles were much greater. $\text{Na}_4\text{Fe}(\text{CN})_6$ made the particles strongly negative, while LaCl_3 made them strongly positive (Fig. 98).

Figure 99 gives the results of a series of experiments at pH 4.0. At pH 4.0 the protein particles, such as gelatin, casein, and albumin, are positively charged without salts, the cataphoretic P.D. being in the neighborhood of 15 millivolts. LaCl_3 , CaCl_2 , and NaCl depressed the P.D. and the more the higher the concentration of the salt, and Na_2SO_4 depressed the cataphoretic P.D. still more rapidly. All these effects of salts on the cataph-

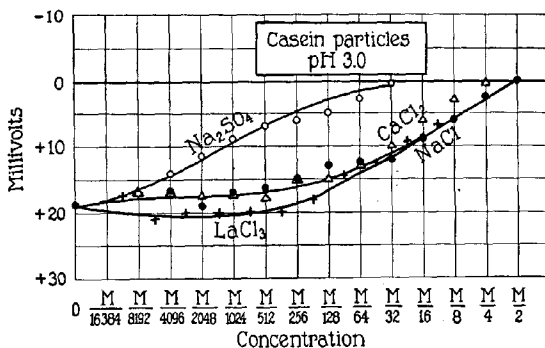


FIG. 100.—Influence of salts on the cataphoretic P.D. of casein particles at pH 3.0.

oretic P.D. are similar to the effects of these salts on the membrane potentials at the same pH. What is, however, different is the effect of $\text{Na}_4\text{Fe}(\text{CN})_6$, which reverses the sign of the cataphoretic charge of the particles in as low a concentration as $\text{M}/65,000$. We shall see later that such a reversal of the sign of charge of proteins by $\text{Na}_4\text{Fe}(\text{CN})_6$ occurs only in the case of the cataphoretic but not in the case of membrane potentials of proteins at a pH of 4.0.

Figure 100 gives the influence of NaCl , CaCl_2 , LaCl_3 , and Na_2SO_4 on the cataphoretic potential of casein particles at pH 3.0. At this pH the influence of $\text{Na}_4\text{Fe}(\text{CN})_6$ can no longer be investigated on account of the chemical changes in the salt.

Without salt the casein particles were positively charged at pH 3.0, the cataphoretic P.D. being about 20 millivolts. All the four above-mentioned salts depressed the P.D. and Na_2SO_4 more rapidly than the three chlorides. This effect of these salts on the cataphoretic P.D. is similar to their effect on the membrane potentials at pH 3.0.

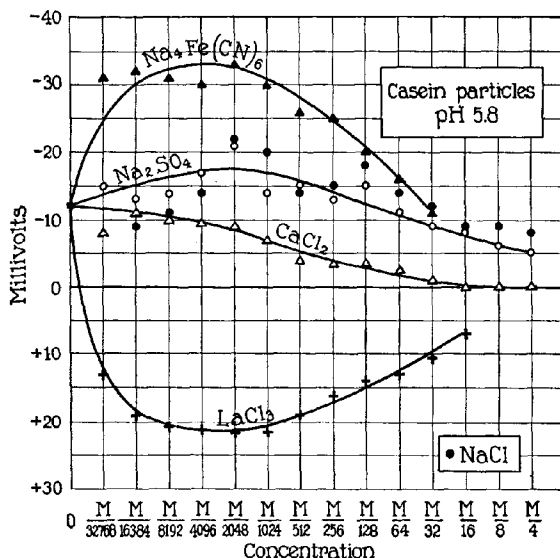


FIG. 101.—Influence of salts on the cataphoretic charge of casein particles at pH 5.8. Without salts the particles are negatively charged. LaCl_3 reverses the sign of charge.

Figure 101 gives the influence of the five salts at a pH of 5.8. Without salt the casein particles were negatively charged, the P.D. being about 12 millivolts. LaCl_3 reverses the sign of charge in as low a concentration as $\text{m}/30,000$, while NaCl , CaCl_2 , and Na_2SO_4 cause no such reversal. $\text{Na}_4\text{Fe}(\text{CN})_6$ causes an enormous increase in the negative charge of the particles, while Na_2SO_4 causes a slight increase. CaCl_2 causes no increase in the cataphoretic P.D. at pH 5.8. Neither the reversal of the sign of charge by LaCl_3 nor the increase of the charge by $\text{Na}_4\text{Fe}(\text{CN})_6$

at pH 5.8 was observed in the case of membrane potentials. These experiments then prove the existence of definite differences between membrane potentials and cataphoretic potentials.

2. To leave no doubt that these differences are real, experiments were made on the effect of $\text{Na}_4\text{Fe}(\text{CN})_6$ and LaCl_3 on the membrane potentials of 3 per cent and 1 per cent solutions of crystalline egg albumin at pH 4.0 and 5.8, respectively. The

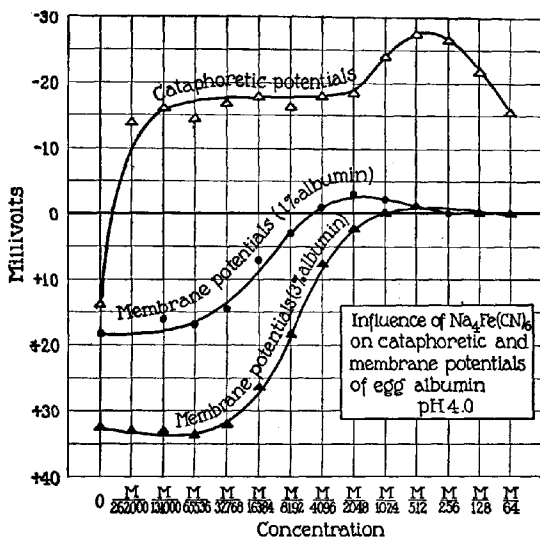


Fig. 102.—Comparison of the influence of $\text{Na}_4\text{Fe}(\text{CN})_6$ on membrane potentials of 1 and 3 per cent solutions of crystalline egg albumin and the cataphoretic potentials of collodion particles coated with crystalline egg albumin at pH 4.0. While low concentrations of the salt reverse the sign of charge of the cataphoretic potentials, no reversal occurs in the case of membrane potentials.

membrane potentials were measured after 18 hours, in the way described in Chap. XI.

Figure 102 gives a comparison of the effects of different concentrations of $\text{Na}_4\text{Fe}(\text{CN})_6$ on the cataphoretic potentials of albumin-coated collodion particles (upper curve) and on the membrane potentials of a 3 per cent and 1 per cent solution of crystalline egg albumin at a pH of 4.0. The ordinates are the

P.D. in millivolts, while the abscissæ are the concentrations of $\text{Na}_4\text{Fe}(\text{CN})_6$ used. When the protein is positively charged, the the P.D. is below the zero line; and when the protein is negatively charged, the P.D. is above the zero line.

Without salt the protein at pH 4.0 is positively charged in both membrane and cataphoretic potentials, but the membrane potential is higher than the cataphoretic potential. While a concentration of $m/200,000$ $\text{Na}_4\text{Fe}(\text{CN})_6$ suffices to reverse the sign of charge of the protein in the case of cataphoretic potentials, no such reversal occurs in the case of the membrane potentials of the 3 per cent albumin solution. In this latter case the salt depresses the P.D. in accordance with Donnan's theory, and at a concentration of $m/1,024$ the P.D. is zero and stays so, even if the concentration of the salt is as high as $m/64$ or above.

A slight reversal seems to occur in the case of the membrane potentials of a 1 per cent solution of albumin at a concentration of $m/2,048$; but this reversal is in reality due to a change in the pH of the protein solution caused by the $\text{Na}_4\text{Fe}(\text{CN})_6$ on standing. Measurements of the pH of the protein solution show that it rises in 18 hours beyond that of the isoelectric point and this causes the reversal of the sign of charge at $m/4,096$ or $m/2,048$. When the concentration of the salt becomes higher the depressing effect of the salt brings the membrane potential again to zero. This reversal of the membrane potential due to a change in the pH did not occur in the 3 per cent protein solution, possibly because the protein acts as a buffer against the pH changes and this buffer action is the greater the higher the concentration of the protein. In the measurements of the cataphoretic potentials no such pH changes occurred, since the measurements of the cataphoretic potentials were made after 20 minutes instead of after 18 hours. In 20 minutes the pH undergoes no material change.

Figure 103 compares the influence of LaCl_3 on membrane and cataphoretic potentials at pH 5.8. Without salt the protein particles as well as the protein solution are negatively charged at pH 5.8. While a low concentration of LaCl_3 , about $m/32,000$ or even less, reverses the sign of charge of the cataphoretic potentials, the salt causes no such reversal in the case of the membrane potentials even in high concentrations. LaCl_3 can only bring

the membrane potentials of a protein solution at pH 5.8 to zero, but cannot reverse their sign of charge.

Near the isoelectric point low concentrations of $\text{Na}_4\text{Fe}(\text{CN})_6$ produce a considerable negative cataphoretic charge, and low concentrations of LaCl_3 produce a considerable positive cataphoretic

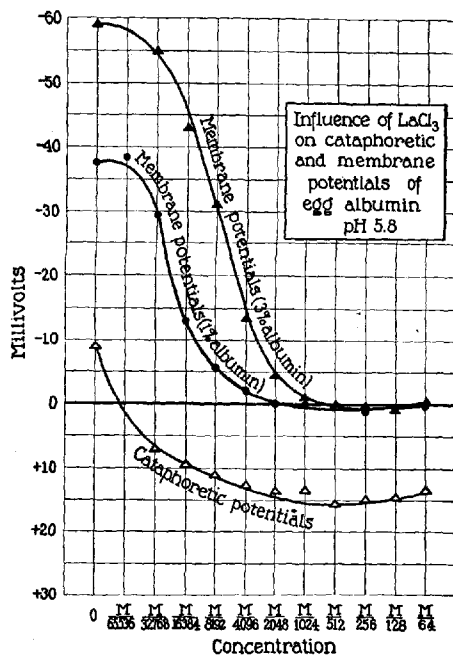


FIG. 103.—Comparison of influence of LaCl_3 on membrane and cataphoretic potentials of albumin at pH 5.8.

retic charge of the albumin particle (see Fig. 109 in the following chapter). Figure 104 shows that they produce no such charge in the membrane potentials of albumin solution at the isoelectric point except that caused by a change in the hydrogen ion concentration of the protein solution by the salt. $\text{Na}_4\text{Fe}(\text{CN})_6$ brings

the solution of crystalline egg albumin in 18 hours to a pH slightly above 4.7.

Previous experiments on the influence of $\text{Na}_4\text{Fe}(\text{CN})_6$ or LaCl_3 on the membrane potentials of gelatin solutions at the isoelectric point had given results similar to the new experiments on egg albumin, and the writer also noticed a tendency of the solution to change its pH. He was not certain at that time that the slight effect of $\text{Na}_4\text{Fe}(\text{CN})_6$ and LaCl_3 on the membrane potentials of isoelectric gelatin was due exclusively to a change of the pH occurring gradually on standing. The new experiments make it more probable that this must have been the case.

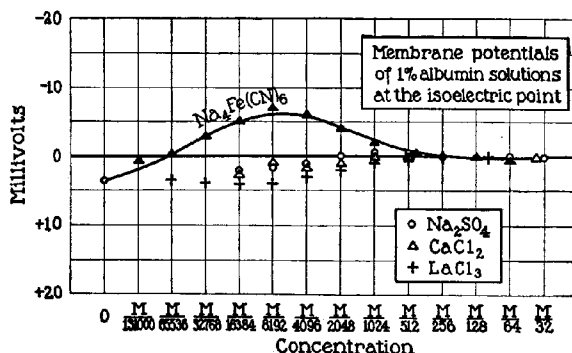


Fig. 104.—Influence of various salts on membrane potentials of albumin solutions at the isoelectric point.

Experiments on the membrane potentials between solid gels of gelatin and aqueous solutions free from gelatin showed that the influence of LaCl_3 and $\text{Na}_4\text{Fe}(\text{CN})_6$ is the same as in membrane potentials of solutions of albumin.

On the other hand, the action of salts of the type of NaCl , CaCl_2 , and Na_2SO_4 is alike in the case of membrane potentials and cataphoretic potentials, since these salts depress the P.D. of both potentials without causing a definite reversal of either. There exists, however, one effect of these latter salts on cataphoretic potentials which does not occur in the case of membrane potentials: Sulphates made the cataphoretic charge of the protein

slightly more negative and CaCl_2 slightly more positive than NaCl . The effect is slight and noticeable only at or near the isoelectric point. This slight effect was not observed in the case of membrane potentials.

We come therefore to the conclusion that a reversal of the sign of charge of protein by low concentrations of salts with tri-valent or tetravalent ions occurs in the case of cataphoretic potentials, but not or practically not in the case of membrane potentials. The fact that such a reversal is brought about in the cataphoretic potentials by low concentrations of LaCl_3 and $\text{Na}_4\text{Fe}(\text{CN})_6$ was corroborated by experiments on two types of phenomena which depend on cataphoretic potentials, namely, on the stability of protein particles and on electrical osmosis through protein films.¹

If we assume the validity of the Helmholtz-Perrin theory of cataphoretic migration, the sign of cataphoretic migration is determined by that film of water which adheres to and moves with the solid particle. This film is usually negatively charged, probably because it has an excess of negative ions which are forced into the film by forces inherent in the water—presumably surface-tension forces. If the two ions of an electrolyte lower the surface tension of water to a different extent, that ion must be driven in excess into the outermost stratum of the double layer (*i.e.*, into the stratum which adheres to and moves with the particle) which has the greater depressing effect on the surface energy. We may conceive that the molecules of water are oriented by such forces at the surface of the water, the oxygen atom forming, as a rule, the outermost, the hydrogen the deeper stratum of the surface.

While thus negative ions are generally forced in excess into the outermost stratum at the surface of the water, the positive ions are in excess in the stratum beneath or in the bulk of the solution. It seems, however, that the force with which cations are driven away from the surface deeper into the water decreases with increasing valency of the cation, so that in the case of salts like LaCl_3 cations are driven with greater force into the outermost stratum of the water than anions, as a consequence of which this stratum becomes positive.

¹ LOEB, J., *J. Gen. Physiol.*, vol. 4, p. 463, 1921-22.

These facts and suggestions probably explain the fact that particles of gelatin chloride of pH 4.0, which are positively charged, assume a negative cataphoretic charge in a weak solution of $\text{Na}_4\text{Fe}(\text{CN})_6$; while particles of Na gelatinate of pH 5.8, which are negatively charged, assume a strong positive charge in a solution of LaCl_3 . We may also understand on this basis why Na_2SO_4 has a tendency to make the cataphoretic charge of the protein particles near the isoelectric point slightly more negative and why CaCl_2 makes the particles a little more positive than does NaCl .

All these facts agree with the idea that the cataphoretic migration is, indeed, determined by the P.D. of an electrical double layer situated entirely in the water and determined at least partly by forces inherent in the water.

3. But this leaves the equally striking fact unexplained that changes in the hydrogen ion concentration affect the cataphoretic P.D. of protein particles similarly as they affect the membrane potentials of protein solutions. Figure 105 gives the influence of acids and alkalis on the cataphoretic P.D. of collodion particles coated with gelatin. These particles were prepared in the following way: To a thick suspension of collodion particles was added enough isoelectric gelatin to make a 1 per cent gelatin solution. The water used for the solution had a pH of 4.7, *i.e.*, the pH of the isoelectric point. The collodion particles remained in the solution overnight. The next day the gelatin solution was heated to about 40°C . to make sure that the gelatin was completely liquefied, and the collodion particles were centrifuged out from the gelatin solution while the latter solution was still warm. The thick sediment of collodion particles which was centrifuged out was suspended in 50 c.c. of water at pH 4.7 and kept as a stock suspension. A few drops of this stock suspension were put into 50 c.c. of acid or alkali of different concentration, shaken up, and left standing for 30 minutes at 24°C . The suspension was then used for the microscopic measurements of the velocity of migration in an electric field. Figure 105 gives the result. At the isoelectric point the P.D. was zero, but both acids and alkalis increased the P.D., the particles being negatively charged in alkali and positively charged in acid, just as in the case of the membrane potentials. When the anion of the acid or the cation of the alkali was monovalent, the cataphoretic P.D. was greater

than when the respective ions were bivalent. This agrees with the valency effect in the case of membrane potentials.

When the concentration of acid or alkali exceeded a certain limit the further increase in concentration diminished the cataphoretic P.D. again and this was also the case with the membrane potentials. It would have been of importance to find out whether the agreement was quantitative, but this was impossible, since we do not know the concentration of protein in the solid

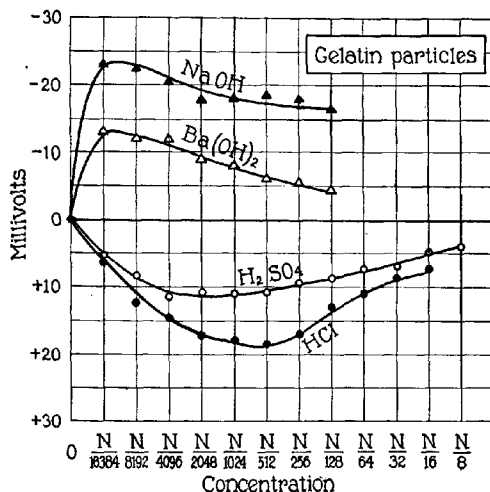


FIG. 105.—Influence of acids and alkalis on the cataphoretic P.D. of collodion particles coated with isoelectric gelatin. Abscissae are the normality of acid or alkali in solution, ordinates the cataphoretic P.D. of the particles in millivolts. The ordinates are above the zero line when the particles are negatively charged, and below when they are positively charged.

particles. The membrane potentials of a 1 per cent solution of protein were for the same pH always greater than the cataphoretic P.D. of solid particles of the same protein.

The question arises: What causes this qualitative agreement between the two potentials in regard to the pH effect? We have a mathematical theory of the effect of acid and alkali on the membrane but, unfortunately, not on the cataphoretic potentials.

4. Freundlich and Rona¹ have noticed a difference between Haber's phase boundary potentials at the boundary of glass and water and the cataphoretic potentials of water against glass. The phase boundary potential depends in this case only on the hydrogen ion concentration of the solution, while other ions, except H and OH, have no direct influence on this potential, as had already been shown by Haber and Klemensiewicz.² The "electrokinetic potential" at the boundary of glass and water measured cataphoretically by Freundlich and Rona showed, however, a striking influence of other ions besides hydrogen and hydroxyl ions, and showed especially the valency effect so characteristic of all cataphoretic potentials. Freundlich and Rona assume that the difference between the two kinds of potential is as follows. The phase boundary potential is the potential difference between the interior of the solid phase and the interior of the liquid and is, therefore, influenced only by those ions of the liquid which can go into the solid phase, and which seem to be in the case of glass only H and OH ions. The electrokinetic potential, however, is in accordance with Helmholtz's theory the potential difference between a film of water adhering to the solid particles and the interior of the water. This P.D. of the double electrical layer is influenced by all the ions of the liquid and the authors assume that adsorption plays the chief rôle in the electrokinetic potentials.

Freundlich and Gyemant³ compared the thermodynamic and electrokinetic potentials between water-immiscible liquids (phenol, guaiacol, benzonitril, and aniline) and aqueous solutions and confirmed the conclusions arrived at by Freundlich and Rona. The thermodynamic potentials between these "oily" liquids and water had been investigated by Beutner⁴ in a series of excellent experiments and his results and conclusions in regard to the origin of these potentials were confirmed by Freundlich and Gyemant. Beutner found that the non-aqueous phase was the

¹ FREUNDLICH, H. and RONA, P., *Sitzb. preuss. Akad. Wiss.*, vol. 20, p. 397, 1920.

² HABER, F. and KLEMENSIEWICZ, Z., *Z. physik. Chem.*, vol. 67, p. 385, 1909.

³ FREUNDLICH, H. and GYEMANT, A., *Z. physik. Chem.*, vol. 100, p. 182, 1922.

⁴ BEUTNER, R., "Die Entstehung elektrischer Ströme in lebenden Geweben," Stuttgart, 1920.

more positively or negatively charged the more soluble the cation or anion of a salt was respectively in the non-aqueous phase. Freundlich and Gyemant found that in the cataphoretic potentials between these four non-aqueous liquids and water the non-aqueous droplets were always negatively charged, even the basic aniline, and the sign of the cataphoretic charge of these water-immiscible droplets could be reversed by polyvalent inorganic cations and by organic cations (*e.g.*, basic dyes). This influence of the cations on the cataphoretic potentials they ascribe to adsorption.

These experiments bring out the difference between thermodynamic potentials and cataphoretic potentials in the cases which Freundlich and his collaborators investigated. The ideas on adsorption can, however, not be used to explain why the membrane potentials of proteins are modified in the same way by H and OH ions as are the cataphoretic potentials, since adsorption, according to Freundlich and Rona, influences only the cataphoretic potentials.

We may state, as a result of our experiments, that the cataphoretic migration and the cataphoretic P.D. of protein particles or of suspended particles coated with a protein are the result of two groups of forces, namely, first, forces inherent in the protein particles (these forces being linked with the membrane equilibrium between protein particles and the outside aqueous solution); and second, forces inherent entirely in the aqueous solution surrounding the protein particles.

The forces inherent in the protein particles are linked with the ionization of the protein, and these forces prevail to such an extent over the forces inherent in the water, that the sense of the cataphoretic migration of protein particles is determined by the forces resulting from the ionization of the protein. J. A. Wilson¹ has suggested that the Donnan equilibrium between the particles and the water determines the cataphoretic P.D. This could be true only for that share in the cataphoretic P.D. which depends on the ionization of the protein. While it is unquestionably true that the ionization of the protein determines the sense of migration of protein particles, it is not equally certain that the Donnan equilibrium is the cause of this connection.

¹ WILSON, J. A., *J. Am. Chem. Soc.*, vol. 28, p. 1982, 1916. "The Chemistry of Leather Manufacture," p. 127, New York, 1923.

CHAPTER XVIII

STABILITY OF SUSPENSIONS OF SOLID PARTICLES
OF PROTEINS, AND PROTECTIVE
ACTION OF COLLOIDS¹

1. INTRODUCTION

While it was formerly held that the forces at the boundary between solids and liquids were purely physical, Langmuir² and Harkins³ have come to the conclusion that these forces are primarily chemical. This viewpoint will be utilized in the problem which forms the object of this chapter, namely, an investigation of the nature of the forces which keep solid particles of proteins in suspension. The large molecule of proteins does not act as a homogeneous unit, and it is necessary to discriminate for our problem between "aqueous" groups, *i.e.*, groups which have a strong chemical affinity for the molecules of water (*e.g.*, carboxyl and amino or imino groups), and "oily" groups (*e.g.*, hydrocarbon groups) which have a stronger affinity for each other than for water. A similar view was expressed by Sheppard.⁴ The molecules of gelatin in aqueous solution may adhere to each other wherever they touch each other with their oily groups; and if the concentration of the gelatin solution is high enough, the whole mass will set to a solid gel. In this case the affinity of the aqueous groups of the gelatin molecule for water need not be, and probably is not, lessened, and when the gelatin sets to a gel the average distance between the molecules of gelatin remains the same as it was in the solution. When, however, gelatin is

¹ The contents of this chapter are based on LOEB, J., *J. Gen. Physiol.*, vol. 5, p. 479, 1922-23.

² LANGMUIR, I., *J. Am. Chem. Soc.*, vol. 39, p. 1848, 1917.

³ HARKINS, W. D., BROWN, F. E. and DAVIES, E. C. H., *J. Am. Chem. Soc.*, vol. 39, p. 354, 1917; HARKINS, DAVIES and CLARK, G. L., *Ibid.*, p. 541.

⁴ SHEPPARD, S. E. and SWEET, S. S., *J. Am. Chem. Soc.*, vol. 44, p. 2797, 1922.

precipitated by a salt the affinity of the aqueous groups for water is diminished and the molecules attract each other over a larger area. The result is a coagulation in which the average distance between the molecules of gelatin is very much less than it was in the solution. This view of the difference between gel formation and precipitation or salting out is supported by the fact observed by the writer that it requires the same high concentration of a salt to cause precipitation of a gelatin solution when it is near complete gel formation as is required when the same solution has a very low viscosity, the temperature in both cases, of course, being equal.

These facts are mentioned because they show that when a solid particle, *e.g.*, collodion, is coated with a film of a protein like gelatin, it is not only *a priori* possible but, perhaps, probable that the affinity of the aqueous groups of the gelatin molecule for water continues to act. It is intended to show in this chapter that the flocculation or precipitation of suspensions of gelatin-coated particles of collodion by salts is the same process of "salting out" by which *solutions* of gelatin in water are precipitated by salts; and it is, moreover, intended to show that the suspensions of gelatin-coated particles of collodion are as unstable at the pH of the isoelectric point of gelatin, namely 4.7, as are the solutions of gelatin, and that *suspensions* of gelatin-coated collodion particles as well as *solutions* of gelatin are stabilized or rendered more soluble at pH 4.7 by the addition of neutral salts. It follows from this that the forces which determine the stability of suspensions of gelatin-coated particles of collodion in water are the forces of chemical affinity between the aqueous groups of the gelatin molecule and water.

This chemical viewpoint is in strong contrast with the purely physical viewpoint, whereby suspended particles are protected against coalescence merely by their electrical double layers, which have their origin primarily in forces inherent in the water itself. On this assumption, the stability of a suspension depends only on the potential difference between the two strata of the electrical double layer surrounding the particle in water, and this P.D. is reduced to zero by comparatively low concentrations of salts, that ion of the salt being effective which has a sign of charge opposite to that of the particle. It will be shown that when

collodion particles are coated with genuine crystalline egg albumin they behave as if the film formation destroyed the affinity of the protein for water in the same way as does high temperature ("boiling"), and in this case the chemical forces of attraction between the aqueous groups of albumin and water play no part in the stability of the suspensions. Such particles can be kept in suspension only by the electrical double layers surrounding the particles, as the physical theory demands.

2. THE NATURE OF THE FORCES WHICH DETERMINE THE STABILITY OF SUSPENSIONS OF GELATIN-COATED PARTICLES OF COLLODION

Suspensions of collodion particles were prepared as described in a previous publication.¹ A small quantity of such particles was put overnight into 100 c.c. of an aqueous solution of 0.1 per cent isoelectric gelatin at a pH of 4.7. The writer has previously shown that under such conditions a solid film of protein is formed on the surface of the collodion.² (It was found important not to use a higher concentration of gelatin than 0.1 per cent, since when a stronger gelatin solution is used, larger gelatin aggregates are formed to which several particles of collodion may stick. Such larger masses will settle rapidly without salt, thus rendering the test futile.) The next morning the (now gelatin-coated) collodion particles were centrifuged from the 0.1 per cent gelatin solution and then enough of the particles of a certain size were put into water of the desired pH to form a creamy suspension. Three drops of such a stock suspension were then put at room temperature overnight into test tubes containing 10 c.c. of a salt solution of a definite pH, to find out which concentration of salts was required for precipitation. The P.D. of the double electrical layer of the gelatin-coated particles is known from the cataphoretic experiments of the preceding chapter.³ In Table LII are given the molar concentrations of different salts required to cause precipitation of the suspension of gelatin-coated collodion particles overnight, at room temperature.

¹ LOEB, J., *J. Gen. Physiol.*, vol. 5, p. 109, 1922-23.

² LOEB, J., *J. Gen. Physiol.*, vol. 2, p. 577, 1919-20.

³ LOEB, J., *J. Gen. Physiol.*, vol. 5, p. 395, 1922-23.

TABLE III.—MINIMAL MOLAR CONCENTRATIONS OF SALTS REQUIRED TO PRECIPITATE SUSPENSIONS OF GELATIN-COATED COLLIDION PARTICLES OVERNIGHT AT ROOM TEMPERATURE

pH	NaCl	CaCl ₂	LaCl ₃	Na ₂ SO ₄	Na ₄ Fe(CN) ₆
	M	M	M	M	M
3.0	2	>2	>1	1	
4.0	>2	>2	>1	1	½
4.7	>2	>2	>1	>½	½
5.8	>2	>2	>1	1	½
11.0	>2	2	...	1	½

The concentration of salts required for precipitation of the gelatin suspension bears no relation to the cataphoretic P.D., first, since the concentrations are far in excess of those required to depress the P.D. to any low value or even zero; and second, since there is no indication of the valency effect which is so strong in the depression of the cataphoretic P.D. by salts. The concentrations of salts required to precipitate the suspensions of negatively charged collodion particles *free from protein* were for NaCl, Na₂SO₄, CaCl₂, and LaCl₃, $m/2$, $m/4$, $m/32$, and $m/2,048$, respectively. At pH 5.8 and 11.0, the gelatin-coated particles of collodion are also negatively charged; yet the concentrations of LaCl₃ required for their precipitation are greater than 1 m , and for CaCl₂ greater than 2 m ; as a matter of fact, the writer is not absolutely certain that these salts precipitate the suspension of the negatively charged gelatin-coated particles at any concentration, though this may be the case. Moreover, Na₂SO₄ and Na₄Fe(CN)₆ are more powerful precipitants for the negatively charged gelatin-coated collodion particles than LaCl₃ or CaCl₂. These results admit only one explanation, namely, that the forces which determine the stability of suspensions of gelatin-coated collodion particles are not the electrical charges of the particles.

The influence of salts on the stability of suspensions of gelatin-coated particles of collodion is especially interesting when the pH of the solution is 4.7, *i.e.*, that of the isoelectric point of gelatin. The cataphoretic measurements show that at the isoelectric point the particles are entirely uncharged. It happens that

at pH 4.7 suspensions of gelatin-coated particles in water free from salt are unstable. At first sight, this would seem to suggest that electrical charges of the particles are required to make suspensions of gelatin-coated particles stable at the isoelectric point of gelatin. This assumption is, however, refuted by the fact that comparatively low concentrations of salts, which leave the particles uncharged, make the suspensions stable. Thus, $m/16,000$ CaCl_2 or $m/16,000$ Na_2SO_4 will suffice to make the suspension of gelatin-coated collodion particles stable at the isoelectric point; NaCl stabilizes the suspension in a concentration of $m/512$. The measurements of the cataphoretic P.D. of gelatin-coated collodion particles at the isoelectric point of gelatin show that neither NaCl , Na_2SO_4 , nor CaCl_2 causes the particles to be charged at pH 4.7, not only in concentrations as low as $m/16,000$ CaCl_2 or Na_2SO_4 , but even in much higher concentrations.¹ Table LIII gives the concentrations of salts required for stabilization at the isoelectric point of gelatin.

TABLE LIII.—CONCENTRATIONS OF SALTS AT WHICH SUSPENSIONS OF GELATIN-COATED PARTICLES OF COLLODION ARE STABLE AT pH 4.7 (ISOELECTRIC POINT OF GELATIN)

	Stable	Precipitated
NaCl	$m/512$ to 2 m (or higher)	0 to $m/1,024$
CaCl_2	$m/16,000$ to 2 m	0
LaCl_3	$m/130,000$ to 1 m	0
Na_2SO_4	$m/16,000$ to $m/2$	0 to $m/32,000$ and also above $m/2$
$\text{Na}_4\text{Fe}(\text{CN})_6$	$m/1,000,000$ to $m/4$	0 also above $m/4$

If, then, the forces which determine the stability of suspensions of gelatin-coated collodion particles are not the potential differences of the electrical double layer surrounding the particles, the question arises: What other forces determine this high degree of stability of these suspensions? The answer is that these forces are the same as those which keep gelatin in solution. It can be shown that the concentrations of salt required for the precipita-

¹ LOEB, J., *J. Gen. Physiol.*, vol. 5, p. 395, 1922-23.

tion of suspensions of gelatin-coated particles are practically identical with those required for the salting out of gelatin from true aqueous solutions. Table LIV gives the minimal concentration of NaCl, CaCl₂, LaCl₃, Na₂SO₄, and Na₄Fe(CN)₆ required to "salt out" 1 per cent solutions of gelatin at pH 3.0, 4.0, 4.7 (isoelectric point), 5.8, and 11.0. The solutions were allowed to stand overnight at room temperature and were prepared in the following way: One gram of originally isoelectric gelatin was dissolved in 100 c.c. of water containing enough HCl or NaOH, respectively, to bring the solution to the desired pH and also the required concentration of salt.

TABLE LIV.—MOLAR CONCENTRATIONS OF SALTS REQUIRED TO PRECIPITATE GELATIN FROM 1 PER CENT SOLUTIONS AT ROOM TEMPERATURE

pH	NaCl	CaCl ₂	LaCl ₃	Na ₂ SO ₄	Na ₄ Fe(CN) ₆
	M	M	M	M	M
3.0	2	>2	> $\frac{7}{8}$	$\frac{1}{2}$	
4.0	>2	>2	> $\frac{7}{8}$	$\frac{1}{2}$ or above	$\frac{7}{16}$
4.7	>2	>2	> $\frac{7}{8}$	$\frac{1}{2}$ or above	$\frac{7}{16}$
5.8	>2	>2	> $\frac{7}{8}$	$\frac{7}{8}$	> $\frac{7}{16}$
11.0	>2	>2	$\frac{7}{8}$	$\frac{7}{16}$

The values in Table LIV are almost identical with the values in Table LII.

It is obvious that the concentrations of salt, *e.g.*, CaCl₂ or LaCl₃, required for precipitation of solutions of gelatin in water are many times greater than would be required if gelatin were in suspension, *i.e.*, if the gelatin particles were prevented from coalescing by the electrical double layers around each particle. Moreover, Tables LII and LIV show that Na₂SO₄ is a better precipitant of gelatin solutions than CaCl₂, even if the gelatin particles are negatively charged, *i.e.*, at pH 5.8 or at pH 11.0. If the gelatin particles were kept in solution by electrostatic charges due to electrical double layers, CaCl₂ should be a better precipitant than Na₂SO₄ when the particles of gelatin are negatively charged, which is, however, not the case. Since the con-

centrations of salts required for the salting out of gelatin from its aqueous solution are almost identical with the concentrations required to precipitate suspensions of gelatin-coated collodion particles, the forces determining the stability of the suspensions must be identical with those determining the stability of true solutions of gelatin, and these latter forces are forces of affinity between solute and solvent.

It can also be shown that the stabilizing effect of salts on suspensions of collodion particles coated with gelatin at the isoelectric point is due to an influence on the affinity of the protein film for water.

It has been shown in Chap. VI that neutral salts increase the solubility of isoelectric gelatin, and the more the higher the valency of either ion of the salt.¹ That we are dealing in this case with ordinary solubility follows from two facts, namely, first, that the time required to dissolve a given mass of solid isoelectric gelatin is diminished upon the addition of salts; and second, that the amount of isoelectric gelatin dissolved in water increases with the addition of salt. Both effects increase with the valency of one of the ions of the salt.

From the practical identity of the influence of salts on the stability of *solutions* of gelatin and of *suspensions* of gelatin-coated collodion particles, it follows that the forces which prevent the coalescence of gelatin-coated collodion particles in water are the strong forces determining the ordinary solubility of gelatin in water, *i.e.*, the chemical affinity between the aqueous groups (carboxyl and amino or imino groups) and the molecules of water.

3. SOLUTIONS OF GENUINE CRYSTALLINE EGG ALBUMIN²

Egg albumin exists in two modifications, "genuine" and "denatured" egg albumin. While genuine crystalline egg albumin is highly soluble in water, denatured egg albumin is practically insoluble. The simplest (but not the only) way to transform genuine egg albumin into its water-insoluble modification is by bringing an aqueous solution of the substance to a sufficiently high temperature. It is possible to show that the forces which keep *genuine* egg albumin in solution are the strong secon-

¹ LOEB, J., *Arch. Neerland. physiol.*, vol. 7, p. 510, 1922.

² LOEB, J., *J. Gen. Physiol.*, vol. 5, p. 479, 1922-23.

dary valency forces determining ordinary solubility, while the forces which keep *denatured* egg albumin in solution or rather suspension are the weak electrostatic forces of repulsion due to the electrical double layer around the particles. We will first discuss the solubility of genuine egg albumin in water.

It is understood that in all the following experiments with genuine egg albumin the temperature is ordinary room temperature not exceeding 24°C., since at temperatures of 60°C. or above crystalline egg albumin may be transformed into its insoluble modification.

Since the particles of genuine isoelectric egg albumin do not migrate at the pH of the isoelectric point, they cannot be kept in solution at that point by electrical charges; moreover, the viscosity of isoelectric solutions of albumin is of so low an order of magnitude that the solutions can contain comparatively few (if any) aggregates. The idea that crystalline egg albumin forms a true solution is also held by Sørensen¹ and is contradicted by no fact.

The concentrations of salts required to precipitate crystalline egg albumin in the neighborhood of the isoelectric point, *i.e.*, at pH 4.0, 4.8, and 5.8, are very high, and show no relation between

TABLE LV.—MINIMAL CONCENTRATIONS OF SALTS REQUIRED TO CAUSE PRECIPITATION IN ABOUT 20 HOURS AT ROOM TEMPERATURE IN 1 PER CENT SOLUTIONS OF ORIGINALLY ISOELECTRIC CRYSTALLINE EGG ALBUMIN AT DIFFERENT pH

pH	NaCl	MgCl ₂	CaCl ₂	LaCl ₃	Na ₂ SO ₄	Na ₄ Fe(CN) ₆
	M	M	M	M	M	M
11.0	1	...	$\frac{1}{4}$...	$>\frac{3}{4}$	$>\frac{3}{8}$
5.8	>2	...	>2	$\frac{1}{2}$	$>\frac{3}{4}$	$>\frac{3}{8}$
4.8						
(Isoelectric point)	>2	...	>2	$<\frac{3}{4}$	$>\frac{3}{4}$	$>\frac{3}{4}$
4.0	>2	...	1	$\frac{1}{4}$	$>\frac{3}{4}$	$>\frac{3}{8}$
3.0	1	...	1	$<\frac{1}{4}$	$<\frac{3}{4}$	
2.0	$\frac{3}{8}$	$\frac{3}{16}$				

¹ SØRENSEN, S. P. L., *Compt.-rend. trav. lab. Carlsberg*, vol. 12, Copenhagen, 1915-17.

the sign of charge of the protein particle and the precipitating ion. At higher hydrogen ion concentrations, *e.g.*, pH 2.0, lower concentrations of salts are sufficient for precipitation. Salts like LaCl_3 have a greater precipitating power for egg albumin at any pH than salts like CaCl_2 (Table LV).

These facts leave no doubt that the solubility of genuine crystalline egg albumin is not determined by double electrical layers surrounding the molecules or particles, but is determined by the forces responsible for true crystalloidal solution, and that the precipitation of genuine egg albumin from its solution is a true "salting out," but is not caused by a diminution of the P.D. of a double layer.

4. THE STABILITY OF SUSPENSIONS OF COLLODION PARTICLES COATED WITH CRYSTALLINE EGG ALBUMIN

Collodion particles coated with genuine crystalline egg albumin do not behave like solutions of genuine egg albumin, but like suspensions of particles of denatured egg albumin; or, in other words, when genuine crystalline egg albumin forms a film on a collodion surface it behaves as if its aqueous groups had ceased to react with water. Suspensions of albumin-coated collodion particles are stable only as long as the cataphoretic P.D. of the particles is above about 12 or 13 millivolts.

Collodion particles were kept overnight in 1 per cent solutions of crystalline egg albumin of pH 4.8, centrifuged off from the solution, and then a milky stock suspension was prepared in water of pH 4.8. Three drops of that suspension were added to 50 c.c. of various concentrations of salt at pH 11.0, 5.8, 4.5, 4.0, and 3.0, and the velocity of migration in an electric field was measured under the microscope.

The results of these measurements are represented graphically in Figs. 106 to 108. The ordinates of the curves representing the influence of salts on the cataphoretic P.D. of the albumin-coated collodion particles are the millivolts calculated from the mobility measurements as described previously; the values are given as negative when the particle bears a negative charge. The curves are practically identical with those for the cataphoretic P.D. of particles of denatured egg albumin (see Figs. 109 to 112), except

at pH 5.8 and 11.0, where the collodion particles coated with egg albumin behave almost like collodion particles free from albumin.

The influence of salts on the stability of suspensions of albumin-coated particles of collodion was tested in the following way: Three drops of the stock suspension of the particles were shaken up in 10 c.c. of various concentrations of salts at different pH and

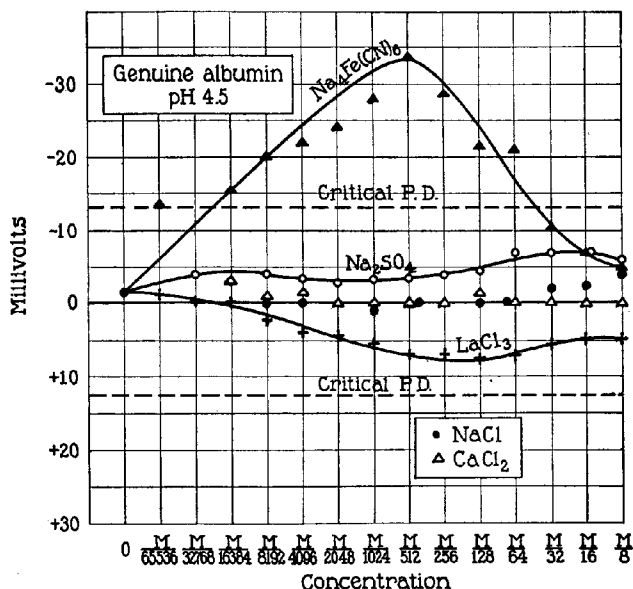


FIG. 106.—Influence of salts on the cataphoretic P.D. of collodion particles coated with a film of crystalline egg albumin at pH 4.5 where the cataphoretic P.D. without salt was about zero. The two broken lines in Fig. 106 and in the following figures with the designation "Critical P.D.," give that P.D. below which the suspensions of the albumin-coated particles are no longer stable. It is obvious that only in solutions of $\text{Na}_4\text{Fe}(\text{CN})_6$ between concentrations of $M/16,000$ and $M/32$ was the suspension stable.

allowed to settle overnight at room temperature. The results are given by the horizontal line, "Critical P.D.," in Figs. 106 to 108. In all salt solutions between that line and the zero line, the particles were generally precipitated overnight. We shall see later that the critical P.D. for the stability of collodion particles

coated with albumin is almost the same as the critical P.D. for suspensions of particles of denatured egg albumin, namely, above 10 to 11 millivolts.

We will now go into some details. In Fig. 106 are given the cataphoretic P.D. of the albumin-coated particles of collodion at pH 4.5, at which the charge was zero. The isoelectric point of

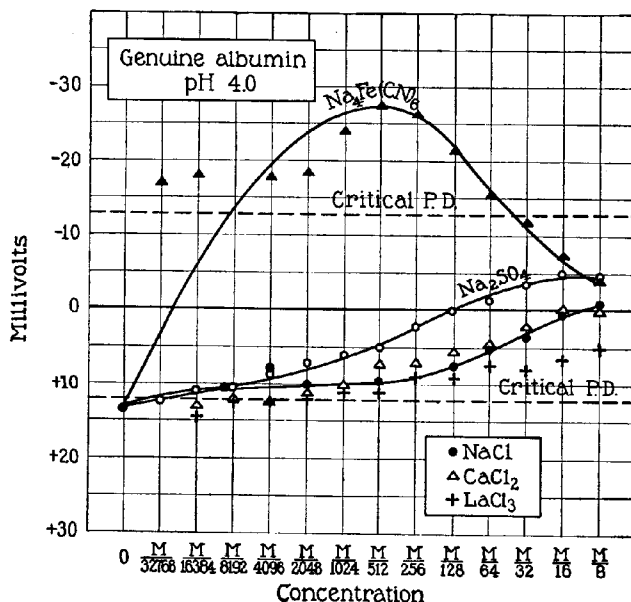


FIG. 107.—Influence of salts at pH 4.0 on the cataphoretic P.D. and stability of collodion particles coated with crystalline egg albumin. Without salt the cataphoretic P.D. is +13 millivolts. $\text{Na}_4\text{Fe}(\text{CN})_6$ brings about a reversal of the sign of charge of the particles. The suspension was stable only in solutions of $\text{Na}_4\text{Fe}(\text{CN})_6$ between $m/8,000$ and $m/32$. In all the other cases the P.D. was below the critical value.

crystalline egg albumin is 4.8, but the cataphoretic P.D. was not zero at pH 4.8 and it was necessary to add a trace of acid to annihilate the cataphoretic migration.

The addition of NaCl , Na_2SO_4 , CaCl_2 , or LaCl_3 did not raise the cataphoretic P.D. to the critical value required for stability. $\text{Na}_4\text{Fe}(\text{CN})_6$, in concentrations of $m/16,000$ or higher, raised the

P.D. to 13 millivolts and above, and the suspension was stable. When the concentration of $\text{Na}_4\text{Fe}(\text{CN})_6$ reached or exceeded $\text{m}/32$, the P.D. was depressed below the critical value and flocculation occurred.

At pH 4.0, the cataphoretic P.D. was above the critical value without salt, but the addition of LaCl_3 , CaCl_2 , NaCl , or Na_2SO_4 depressed the P.D. below that of the critical value, and flocculation occurred (Fig. 107). Stable suspensions were obtained in

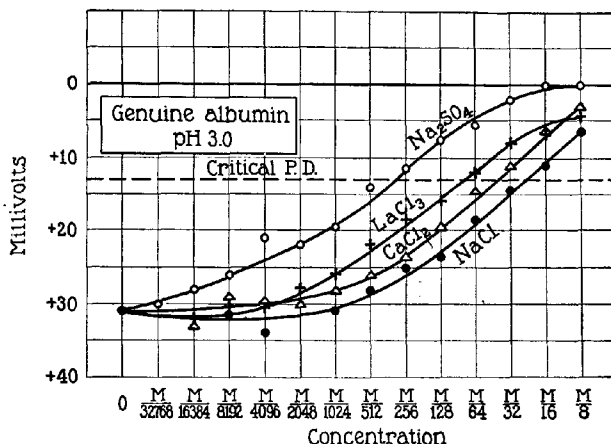


FIG. 108.—Influence of salts on cataphoretic P.D. and stability of albumin-coated particles of pH 3.0. Without salt the cataphoretic P.D. is about 31 millivolts and the particles are positively charged. The suspension is stable as long as the concentration of the salt is not too high. Flocculation occurs when the values for the P.D. fall between the line for critical P.D. and the zero line.

solutions of $\text{Na}_4\text{Fe}(\text{CN})_6$ in concentrations between $\text{m}/8,000$ and $\text{m}/32$, because in these solutions the cataphoretic P.D. was above 13 millivolts.

At pH 3.0 the P.D. was over 30 millivolts without salt and the suspension was stable (Fig. 108). The addition of high concentrations of salts was required to depress the P.D. below the critical value of 13 millivolts. $\text{m}/16$ NaCl , $\text{m}/32$ CaCl_2 , about $\text{m}/64$ LaCl_3 , and $\text{m}/256$ Na_2SO_4 were required to cause flocculation.

Hence, collodion particles coated with genuine egg albumin form stable suspensions only by virtue of their electrical double layers; as soon as the P.D. of the double layer falls below a critical value, the suspension is no longer stable. The particles attract each other in the same way as do the particles of denatured egg albumin when the P.D. falls below the critical value of about 12 millivolts.

It is difficult to understand why genuine egg albumin when it forms a solid film on collodion particles should lose its solubility in water and behave in that respect like boiled egg albumin, yet the fact that albumin is denatured when forming a film is supported by the observations of Ramsden.¹ This author found that proteins have a tendency to form a solid film at the surface of liquids on account of their lowering the surface tension of the water; but he also found that these proteins (or, perhaps, more correctly certain proteins) undergo an irreversible coagulation in this case. Applied to crystalline egg albumin it would mean that genuine egg albumin is denatured when it forms a film.

Herzfeld and Klinger,² as well as Wicchowski,³ found that mechanical grinding of a dry powder of soluble blood albumin renders the albumin insoluble.

The mechanism of denaturation is unknown. Might it be possible that the albumin molecule of the film is oriented in such a way as to render ineffective the action of the groups with a high affinity for water? The observations of Langmuir⁴ leave no doubt that the molecules of surface films are definitely oriented.

Whatever the explanation may be, the fact remains that the influence of electrolytes on the cataphoretic P.D. is practically the same for collodion particles coated with gelatin or with genuine crystalline egg albumin, while the influence of salts on the stability of suspensions of the two types of particles is entirely different. This difference finds its explanation in the fact that the forces determining the stability of suspensions of gelatin-coated particles in water are the strong chemical forces acting in true

¹ RAMSDEN, W., *Z. physik. Chem.*, vol. 47, p. 336, 1904.

² HERZFELD, E. and KLINGER, R., *Biochem. Z.*, vol. 78, p. 349, 1917.

³ WIECHOWSKI, W., *Biochem. Z.*, vol. 81, p. 278, 1917.

⁴ LANGMUIR, I., *J. Am. Chem. Soc.*, vol. 38, p. 2221, 1916; vol. 39, p. 1848, 1917.

solubility, while the forces determining the stability of suspensions of albumin-coated collodion particles are essentially the weak electrostatic forces of the double electrical layer surrounding the particle.

5. THE STABILITY OF SUSPENSIONS OF PARTICLES OF DENATURED EGG ALBUMIN

The conditions for the stability of suspensions of particles of denatured egg albumin are practically identical with those for the stability of suspensions of particles of collodion coated with genuine egg albumin. This supports the idea that genuine egg albumin, when forming a film on collodion, undergoes a change whereby its affinity for water no longer seems to exist.

Careful experiments on the flocculation of denatured egg albumin have been made before, especially by Chick and Martin,¹ but the cataphoretic P.D. of the particles was not measured, and we are here concerned with the relation between that P.D. and the stability of suspensions.

Suspensions of denatured egg albumin were prepared in the following way:² A 1 per cent solution of isoelectric crystalline egg albumin (pH 4.8) was heated to 90°C. and the coagulated mass was allowed to settle. It was then ground in a mortar with a small amount of water to a milky suspension. One drop of a sufficiently concentrated stock suspension was put into 10 c.c. of a solution of different salts at different pH, and the solution was allowed to stand overnight at room temperature.

In order to bring the cataphoretic P.D. of the particles of (boiled) denatured egg albumin to zero, the surrounding solution had to have a pH of about 5.0. Figure 109 gives the influence of NaCl, Na₂SO₄, CaCl₂, LaCl₃, and Na₄Fe(CN)₆ on the cataphoretic P.D. Flocculation occurred in all concentrations of NaCl, Na₂SO₄, and CaCl₂. Only in certain concentrations of Na₄Fe(CN)₆ and LaCl₃ was the suspension stable and these concentrations were, in the case of Na₄Fe(CN)₆, between M/65,000 and M/16. Figure 109 shows that inside these concentrations the cataphoretic P.D. was above 10 millivolts. The suspension of particles of denatured egg albumin was stable in concentrations

¹ CHICK, H. and MARTIN, C. J., *J. Physiol.*, vol. 45, p. 261, 1912-13.

² LOEB, J., *J. Gen. Physiol.*, vol. 5, p. 505, 1922-23.

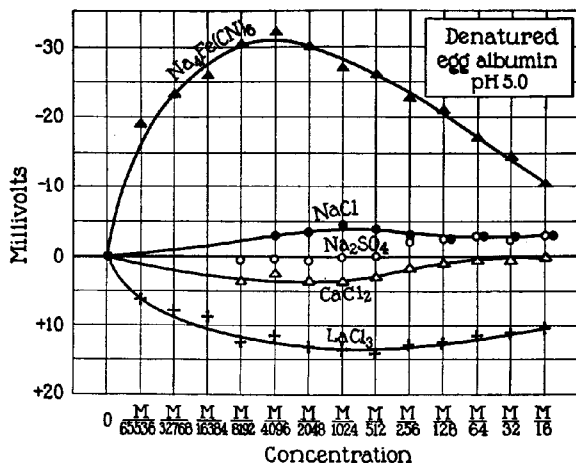


Fig. 109.—Influence of salts on the cataphoretic P.D. of particles of denatured egg albumin near the isoelectric point.

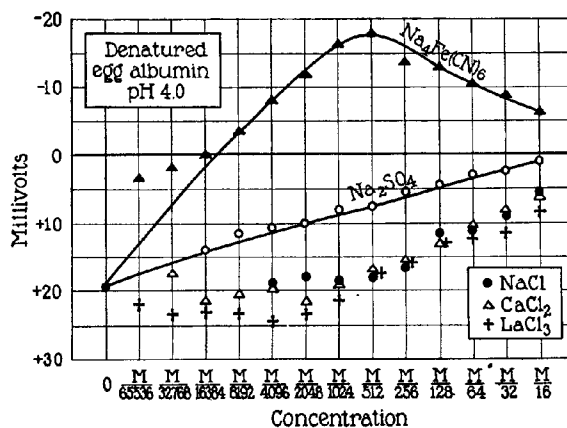


Fig. 110.—Influence of salts on the cataphoretic P.D. of particles of denatured egg albumin at pH 4.0.

of LaCl_3 between $m/2,000$ and $m/16$, and in this case the P.D. was also above the critical level of about 12 millivolts.

Figure 110 gives the influence of the five salts on the cataphoretic P.D. of denatured egg albumin at pH 4.0. At this pH the cataphoretic P.D. of the particles was about 20 millivolts without salt, and since this is above the critical value for P.D. the suspension was stable. The addition of a trace of $\text{Na}_4\text{Fe}(\text{CN})_6$, $m/8,000$ or less, brought the P.D. to about zero and flocculation occurred. The addition of more $\text{Na}_4\text{Fe}(\text{CN})_6$ reversed the sign

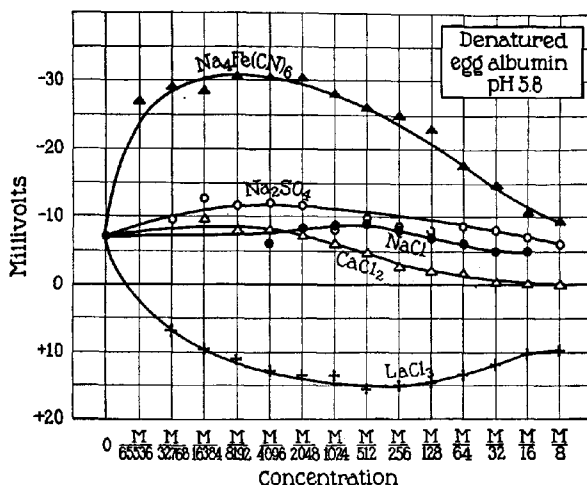


Fig. 111.—Influence of salts on the cataphoretic P.D. of particles of denatured egg albumin at pH 5.8.

of charge and the P.D. increased. At $m/2,048$ the P.D. was about 12 millivolts and the suspension was stable. The other salts depressed the P.D. below the critical value in the following concentrations: NaCl $m/16$, CaCl_2 $m/32$, LaCl_3 below $m/16$, and Na_2SO_4 $m/1,024$; and in these and higher concentrations caused flocculation.

Figures 111 and 112 give the influence of salts on the cataphoretic P.D. of particles of denatured egg albumin at pH 5.8 and 3.0. From these figures, the concentration where flocculation occurs in each of the salts can be predicted, since the suspension is stable

only when the P.D. is above 10 to 12 millivolts. The maximal concentration where the suspension was stable and the minimal concentration where flocculation occurred are given in Table LVI.

The figures in Table LVI show that the suspension of particles of denatured crystalline egg albumin no longer remains stable when the P.D. falls below about 9 millivolts, while it is always stable when it is above 10 to 12 millivolts. It must also be remembered that the measurements of the cataphoretic P.D. are accurate only within ± 2 millivolts.

It follows from this that the stability of suspensions of denatured crystalline egg albumin depends on the P.D. surrounding

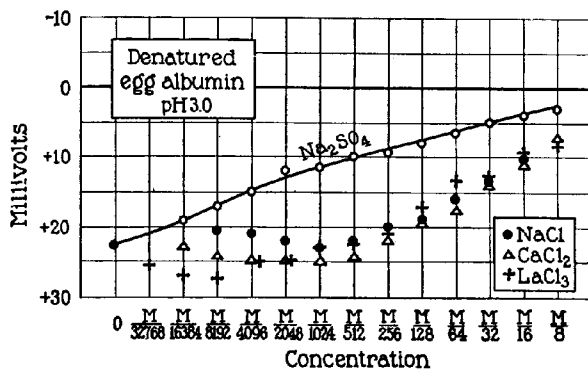


FIG. 112.—Influence of salts on the cataphoretic P.D. of particles of denatured egg albumin at pH 3.0.

each particle. There is, however, one statement to be added. At pH 4.0, 5.8, and 5.0, there occurs a suspension in concentrations of CaCl_2 and NaCl of $m/2$ or above. Since at these high concentrations the P.D. is very low in water, we must conclude that the salt depresses the cohesive forces between the particles, or increases the forces of attraction between water and albumin, so that the particles cannot coalesce even if the P.D. around each particle is zero. Northrop and De Kruif¹ observed a similar phenomenon in their experiments on the influence of salts on bacterial suspensions, and they proved by direct measurements

¹ NORTHROP, J. H. and DE KRUIF, P. H., *J. Gen. Physiol.*, vol. 4, pp. 639, 655, 1921-22.

that the cohesive forces between bacteria may be sufficiently diminished by high concentrations of salt solutions, so that no agglutination occurs between the bacteria even if the P.D. between the bacteria is low or zero.

TABLE LVI.—MINIMAL CONCENTRATIONS AND CATAPHORETIC P.D. AT WHICH SUSPENSIONS OF DENATURED CRYSTALLINE EGG ALBUMIN WERE STABLE OR FLOCCULATED

pH		Stable		Complete precipitation	
		Concentration	P.D. in millivolts	Concentration	P.D. in millivolts
11.0	CaCl ₂	m/256	10	m/16	6
	NaCl.....	m/4 to 0	24 to 9		
	Na ₂ SO ₄	m/4 to 0	31 to 10	1m	<10
5.8	NaCl.....	m/256	8	m/128	7
	CaCl ₂	m/4,096	8	m/2,048	7
	LaCl ₃	m/2,048	13	m/32,000	7
	Na ₂ SO ₄	m/4,096	12	m/512	9
	Na ₄ Fe(CN) ₆	m/4	<9
5.0	NaCl.....	0 to m/4	<3
	CaCl ₂	0 to m/8	<3
	Na ₂ SO ₄	0 to m/2	<3
	LaCl ₃	m/2,048 to m/16	13 to 10	m/32,000 to 0	8 to 6
	Na ₄ Fe(CN) ₆	m/65,000 to m/16	19 to 10.5	0	5
4.0	NaCl.....	m/32	9	m/16	6
	CaCl ₂	m/128	13	m/32	8
	LaCl ₃	m/65,000 to m/16	22 to 9		
	Na ₂ SO ₄	m/16,000	14	m/1,024	8
	Na ₄ Fe(CN) ₆	m/4,096 to m/2,048	12 to 8	m/8,192	3
3.0	NaCl.....	m/16	10.5	m/4	
	CaCl ₂	m/16	11.0	m/4	
	LaCl ₃	m/16	9.5	m/4	
	Na ₂ SO ₄	m/256	9.5	m/128	8

6. THE INFLUENCE OF SALTS ON THE HEAT COAGULATION OF DENATURED EGG ALBUMIN

When crystalline egg albumin is heated it undergoes a change whereby it becomes insoluble. Its molecules upon colliding will adhere to each other and form aggregates and these aggregates may further coalesce upon colliding, provided the cataphoretic P.D. is below that of the critical value for coalescence, which in

the preceding paragraph was shown to be about 9 millivolts. When the cataphoretic P.D. is above this critical value no such coalescence will occur and the suspension will be stable. But the average size of the particles will be the smaller the higher the P.D., because the probability of the particles approaching each other with sufficient kinetic energy to break through the barrier of electrostatic repulsion becomes the smaller the higher the cataphoretic P.D. This will show itself in the appearance of the solution. When the P.D. is very high, the solution must remain clear as water, because there may be aggregates of, at the utmost, a few molecules, but no coalescence of such aggregates can occur. When the P.D. is a little less, a small percentage of particles may possess a kinetic energy sufficient to break through the barrier of electrostatic repulsion and some coalescence of aggregates may occur. Such suspensions may appear slightly bluish opalescent. Upon further diminution of the P.D. the relative percentage of coalescence will increase, the suspension will appear gray, finally milky, and when the P.D. falls below the critical value, coalescence will be so general that the majority of the colliding aggregates will coalesce and flocculation will occur, since the rate of settling of a suspension depends on the relative size of the particles.

Now the cataphoretic P.D. of the particles is influenced by the hydrogen ion concentration and by other salts, and the influence of the salts can be gathered from the curves in Figs. 109 to 112.

The experimental procedure was as follows: 7 c.c. of water of pH 4.8 (this pH being the isoelectric point of crystalline egg albumin) were added to 2 c.c. of 1 per cent solution of isoelectric crystalline egg albumin (of course, also of pH 4.8) and then 1 c.c. of a salt solution containing different salts of different concentration, but always of pH 4.8, was added. The test tubes containing the 10 c.c. of the mixtures were put into boiling water until the liquid in the test tubes reached a temperature of 90°C. and then the test tubes were taken out of the water bath and allowed to cool to room temperature. Table LVII gives the appearance of the various mixtures after standing overnight.

Without salt the cataphoretic charge of the aggregate is zero and flocculation occurs. When either NaCl, Na₂SO₄, or CaCl₂ is

TABLE LVIII.—INFLUENCE OF HCl ON HEAT COAGULATION OF CRYSTALLINE EGG ALBUMIN IN AQUEOUS SOLUTION. TEN CUBIC CENTIMETERS OF 0.2 PER CENT ALBUMIN (NEARLY ISOELECTRIC), CONTAINING VARIOUS CONCENTRATIONS OF 0.1 N HCl, HEATED TO 90°C.

Cubic centimeters 0.1 N HCl in 10 c.c. of 0.2 per cent albumin	0	0.01	0.02	0.03	0.04	0.05	0.1	0.2	0.4	0.8	1.6	3.2
	Coagulated	Very opaque	Very opalescent	Clear but slightly opalescent	Very clear, like water							
Appearance of solution.....												

in the solution, flocculation will always occur, since Fig. 106 shows that these salts raise the P.D. little or not at all. When, however, $\text{Na}_4\text{Fe}(\text{CN})_6$ or LaCl_3 is added, not only is there a suspension formed, but that suspension becomes almost as clear as water at that concentration of these salts at which the P.D. of denatured egg albumin is high. Thus the suspension of denatured egg albumin remains clear though slightly bluish in appearance in concentrations of LaCl_3 between $m/2,800$ and $m/40$. Now in this region the P.D. is a maximum at the isoelectric point, being nearly 15 millivolts. At $m/25$ and at $m/10,000$ the suspension is still stable but opaque, *i.e.*, the particles are larger but not large enough to settle rapidly. At these concentrations of LaCl_3 the P.D. was about 10 millivolts, *i.e.*, it was just above the critical value of 9 millivolts. In concentrations of $m/20,000$ or less LaCl_3 flocculation occurred, since the P.D. was below the critical value of 9 millivolts.

Acid acts in the same way. When traces of acid are added to isoelectric albumin, heat coagulation is prevented, but the appearance of the solution of egg albumin after heating depends on the P.D. of the particles in the way described. Ten cubic centimeters of an aqueous 0.2 per cent solution of almost isoelectric crystalline egg albumin and contain-

ing varying amounts of 0.1 N HCl were put into test tubes, and these test tubes were put into boiling water until the temperature of the albumin rose to 90°C. Then the test tubes were allowed to cool to room temperature and the appearance of the solution was noticed. Table LVIII gives the result.

When the 10 c.c. contained 0.01 c.c. of 0.1 N HCl the protein remained practically isoelectric (pH 4.8), the P.D. remained below that of the critical point, and hence flocculation occurred upon heating. When 0.02 c.c. of 0.1 N HCl was added, coagulation no longer occurred, but enough particles could coalesce because the P.D. was not very high. Only when 0.05 c.c. or more acid was added did the cataphoretic P.D. become high enough to keep the solution clear on boiling. When the concentration of

TABLE LIX.—HEAT COAGULATION OF CRYSTALLINE EGG ALBUMIN

pH		No coagulation		Coagulation	
		Concentration	P.D. in millivolts	Concentration	P.D. in millivolts
11.0	NaCl.....	0 to m/2	24 to 9		
	CaCl ₂	0 to m/512	31 to 12.5	m/128 to 2 m	8.5 and less
	Na ₂ SO ₄	0 to m/8	31 to 12	1 m	
	Na ₄ Fe(CN) ₆	0 to m/8	29 to 9.5	m/2	
5.8	NaCl.....			m/16 and above	5 and less
	CaCl ₂			m/512 to 1 m	5 and less
	Na ₂ SO ₄			m/16 to m/2	7 and less
	Na ₄ Fe(CN) ₆			m/4	
4.0	NaCl.....			m/64 to 2 m	11 and less
	CaCl ₂			m/128 to 2 m	13 and less
	LaCl ₃			m/4	
	Na ₂ SO ₄			m/1,024 to 1 m	8 and less
3.0	NaCl.....	m/32	13.5	m/4	
	CaCl ₂	m/32	14.0	m/8 to 2 m	8 and less
	LaCl ₃	m/32	13.0	m/4	
	Na ₂ SO ₄	m/512	10.0	m/128	8 and less
4.8	NaCl.....			At all concentrations	About 3 or less
	CaCl ₂				
	Na ₂ SO ₄				
	LaCl ₃	m/2,500 to m/26	13 to 11		
	Na ₄ Fe(CN) ₆	m/2,500 to m/20	30 to 14		

acid was sufficiently increased so as to bring the cataphoretic P.D. down again below the critical value, flocculation occurred again.

Table LIX compares the maximal concentrations of salts at which solutions of genuine crystalline egg albumin no longer flocculate upon heating to 90°C. and the minimum concentrations required for flocculation. The table also gives the cataphoretic P.D. of denatured particles of white of egg at these concentrations.

7. PROTEINS AS PROTECTIVE COLLOIDS

In his experiments on anomalous osmosis the writer showed that when collodion membranes are filled with a 1 per cent solution of a protein, such as gelatin, crystalline egg albumin, casein, or oxyhemoglobin, there is formed overnight inside the membrane a durable film of solid protein which cannot be washed away even if the interior is rinsed out as often as ten or twenty times with warm water.¹ This film betrays itself by its color in the case of oxyhemoglobin. The forces which make the film adhere to the collodion must be very strong, but they do not depend upon the ionization of the protein, since the films are formed no matter whether the protein is at the isoelectric point, or whether it is on the acid side of the isoelectric point. It is, however, not formed when the protein is on the alkaline side of the isoelectric point. The forces which cause the film formation must be those forces of secondary valency responsible for phenomena of adhesion and cohesion in general.

This film formation is responsible for the so-called protective action of certain colloids. Zsigmondy and his collaborators showed that suspensions of colloidal gold, which were precipitated by low concentrations of salts, were protected against such precipitation when the gold particles were suspended in a gelatin solution; and to make the work quantitative he introduced the term "gold number," defining it as that number of milligrams of protective substance which is just sufficient to prevent a definite degree of agglutination of the gold particles caused by the addi-

¹ LOEB, J., *J. Gen. Physiol.*, vol. 2, p. 577, 1919-20.

tion of 1 c.c. of 10 per cent NaCl to 10 c.c. of suspension of gold particles.¹

Gelatin was found to be especially active as a protective colloid, while egg albumin—which Zsigmondy did not purify by crystallization—had little protective action. The experiments reported in this chapter give an explanation of why gelatin is a good protective colloid and why crystalline egg albumin is not. Suspensions of collodion particles not treated with protein are precipitated by low concentrations of salts because the particles are only kept in suspension by virtue of their double electrical layers, the P.D. of which is brought below the critical value by comparatively low concentrations of salts. When collodion particles are put into a solution of gelatin, a gelatin film is formed at the surface, the molecules of which retain the high affinity of gelatin for water, and this affinity is not destroyed by even very high concentrations of salts. Consequently, the stability of the suspension of gelatin-coated collodion particles no longer depends on the double electrical layer which determined the stability of the collodion particles before they were coated with protein, and which is reduced below the critical value by relatively low concentrations of salts.

When genuine crystalline egg albumin forms a film around a collodion particle, the albumin loses its high affinity for water and behaves like denatured egg albumin, inasmuch as its affinity for water molecules is considerably diminished. Collodion particles coated with egg albumin depend therefore chiefly on the electrical double layer surrounding each particle and hence will be precipitated by low concentrations of salts.

If we take the effects of the hydrogen ion concentration into consideration we can, however, single out certain cases where even a film of crystalline egg albumin has some protective action on suspensions of collodion particles. Thus, a low concentration of LaCl_3 (about $\text{m}/4,000$) suffices to flocculate a suspension of collodion particles free from protein at pH 4.0 or 3.0. When, however, the particles are coated with egg albumin or casein, the concentration of LaCl_3 required for that purpose is much higher, about $\text{m}/32$ or even higher, since at the pH mentioned the particles are positively charged and the P.D. is diminished by the

¹ ZSIGMONDY, R., "Kolloidchemie," pp. 173, 358, Leipsic, 1918.

Cl ion instead of by the La ion. A second protective effect is noticed at pH 4.0 or pH 4.8 in high concentrations of CaCl_2 , $M/2$ or somewhat higher, when the particles are coated with casein or albumin.

Casein has generally little or no protective action, since the stability of casein-coated collodion particles depends on the cataphoretic P.D. which is depressed below the critical value by low concentrations of salts. In certain cases, however, salts, especially CaCl_2 in high concentrations, can keep the particles in suspension even if the P.D. is zero.

Experiments with collodion particles coated with edestin showed that this latter protein is of very little use as a protective colloid.

These experiments permit us to define the conditions for a general protective action of colloids, such as that by gelatin. Protective colloids must first be capable of forming durable films on the surface of the particles to be protected, and second, the molecules constituting the film must have a higher attraction for the molecules of the solvent (*e.g.*, water) than for each other; in other words, they must possess true crystalloidal solubility. Those who refuse to believe that proteins may form true solutions will find it difficult to explain the mechanism of the protective action of such colloids as gelatin.

CHAPTER XIX

MEMBRANE EQUILIBRIUM AND PEPTIZATION

By "peptization" is meant the diminution of the size of aggregates, and peptization is therefore the reverse of flocculation or coalescence of aggregates. The mechanism of the diminution of size of aggregates is not always the same. In the case of the action of pepsin on boiled white of egg we are dealing with a process of hydrolysis resulting in the transformation of the insoluble compound into smaller products possessing true solubility. In other cases the influence on solubility may be due to ionization. Thus, when the solubility limit of gelatin at the isoelectric point is exceeded and an opaque suspension exists, the solution may clear up when the solubility is increased by the addition of a salt, especially with an ion of higher valency. We will here consider a case where the peptization is due to a membrane equilibrium between particle and surrounding solution, namely, in the case of the peptization of granules of originally isoelectric, insoluble casein by acid.

Since isoelectric casein is practically insoluble in water it is easy to study the mechanism of solution of granules of casein in aqueous solutions of acid and alkali. The mechanism is entirely different in the two media. In an alkaline solution, *e.g.*, NaOH, casein granules dissolve very much as do particles of sodium oleate, the solution of which is accompanied by phenomena of spreading. According to Quincke, such phenomena of spreading are due to a sudden lowering of surface tension between the surface layer of soap and water, whereby projecting small particles of the surface are torn off so that the surface of the granules soon becomes smooth. This happens in the case of casein granules in alkali.

The forces which drive the Na caseinate into solution are not the forces of the Donnan equilibrium. If this were the case the rate of solution of the granules should reach a maximum at a

pH of between 10.0 and 12.0 and should then diminish. As a matter of fact the rapidity of solution increases indefinitely with the pH of the NaOH. In $m/2$ NaOH the solution of the granule occurs almost instantaneously. This agrees with the fact that solutions of Na caseinate in water require very high concentrations of NaCl or LiCl or NH_4Cl for precipitation.

A Na caseinate solution of pH 7.0 was prepared containing 2 gm. of originally isoelectric casein in a 100-c.c. solution.¹ Five cubic centimeters of this solution were added to 5 c.c. of solutions of different salts also of pH 7.0. No precipitation was observed when the concentration of NaCl in the caseinate solution was $2\frac{1}{2}$ M or less, or that of LiCl was $3\frac{1}{4}$ M, or that of NH_4Cl was 2 M.

When, however, isoelectric granules of casein are put into solutions of HCl, the forces of cohesion between the molecules of casein are so great that no solution is possible until the molecules are forced apart mechanically by the hydrostatic pressure of the water which is driven into the particles on account of the Donnan equilibrium.

This can be proved by microscopic observation of the mechanism of the solution of solid particles of originally isoelectric casein in solutions of acids of different concentration. It was found that the particles of casein swell in a solution of HCl, becoming more and more transparent the more they swell, and that when the swelling has reached a certain stage, the particles disappear—they are dissolved. When in the swollen stage, slight agitation may make them fall apart. T. B. Robertson had suggested such a mechanism for the solution of Na caseinate, but the mechanism of solution in this latter case seems to be different. There is no doubt, however, that the swelling of casein particles is a necessary prerequisite for the solution of casein-acid salts, since such particles are only dissolved when their swelling exceeds a definite limit.

The method of procedure was as follows: A small number of granules of isoelectric casein of the same size (going through a sieve with mesh 100 but not through a sieve with mesh 120) were put into 50 c.c. of water containing different quantities of different acids and kept at 24°C. At various intervals, *i.e.*, after 15 minutes, and after 1, 6, and 24 hours, the diameter of

¹ LOEB, J. and LOEB, R. F., *J. Gen. Physiol.*, vol. 4, p. 187, 1921-22.

about 15 grains was measured with a micrometer under a microscope and the average diameter calculated. The particles were not stirred, and care was taken to avoid their breaking into smaller fragments. The averages after 1 hour are plotted in Fig. 113. The abscissæ are the logarithms of the concentrations of acid of the aqueous solution; the ordinates are the average

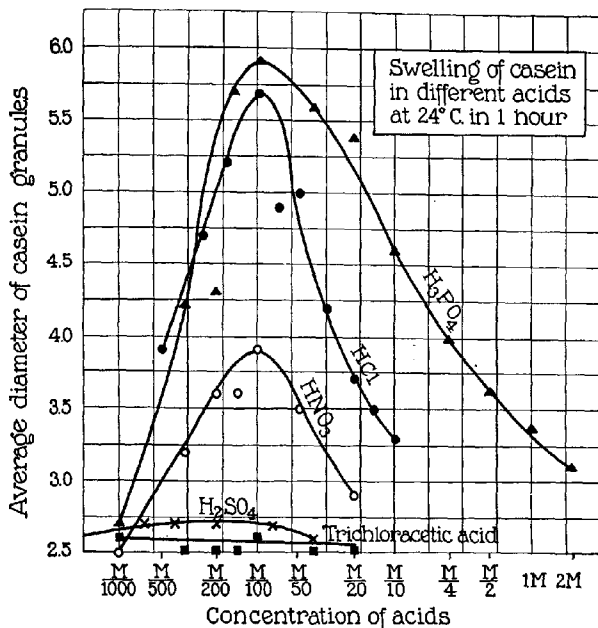


FIG. 113.—Influence of different acids on the swelling of casein.

diameters of the particles. It is obvious that the average diameter of the particles increases at first with the increase of the concentration of the acid, reaching a maximum at about pH 2.0 of the outside solution, and with a further increase in the concentration of the acid the swelling becomes less again.

Figure 114 gives the measurements of the same particles after 24 hours. At this time all the particles in the region of greatest

solubility for HCl and for H_3PO_4 , i.e., between pH of the outside solution of 1.8 and 2.9, had completely dissolved and could no longer be measured.

Figure 114 shows another fact, namely, that the rate of swelling is not the same in different acids. It is about the same in HCl and H_3PO_4 (for the same pH) but decidedly less in HNO_3 and

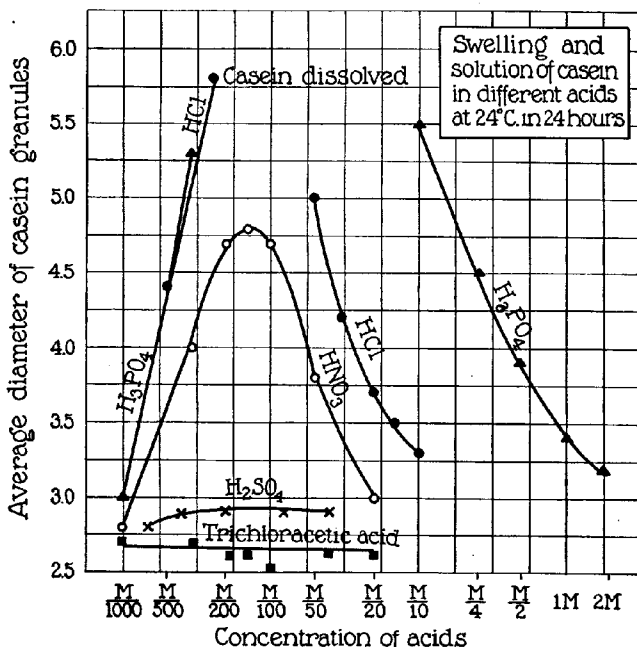


Fig. 114.—Connection between swelling and solution of casein particles.

still less in H_2SO_4 and trichloroacetic acid. It was found that the rate of solution of casein in these different acids followed closely the rate of swelling. It took longer to dissolve casein in HNO_3 , than it did in HCl (at $20^\circ\text{C}.$); and the casein was practically insoluble in H_2SO_4 and trichloroacetic acid in 24 hours.

In this latter case, the forces of cohesion between the oily groups of the casein molecules were increased by the anion of the

acid. The influence of the anion of gelatin-acid salts on the cohesion of the particles of a solid gel is apparently much smaller than the influence of the anion on the cohesion of particles of casein-acid salts. The forces of cohesion in the case of casein sulphate and casein trichloracetate seem to be so great that they cannot be overcome by the osmotic pressure due to the Donnan equilibrium; and hence, no swelling (and as a consequence no

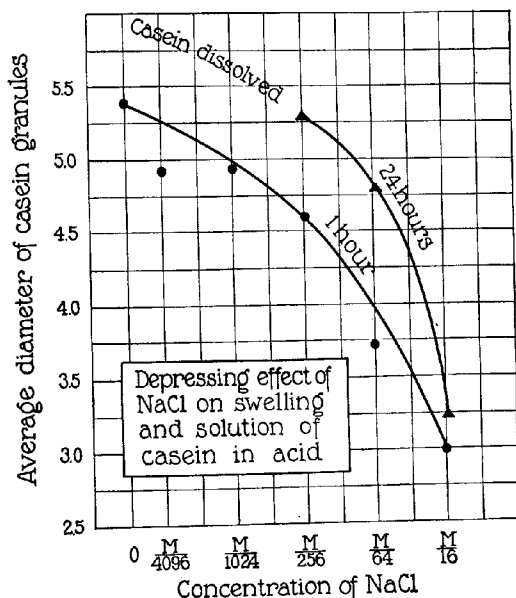


FIG. 115.—Depressing action of NaCl on swelling and solution of casein in acid.

peptization or solution) of solid casein is possible in H_2SO_4 or trichloroacetic acid. The influence of valency on the Donnan equilibrium is the same in the case of the swelling of casein and of gelatin; what is different is the influence of certain ions on the forces of attraction of the oily groups for each other.

Procter and Wilson have shown that the theory of the Donnan equilibrium explains the depressing effect of a salt on the swelling

TABLE LX.—AMOUNT OF CASEIN DISSOLVED AT 20°C. IN HCl OF DIFFERENT pH

pH.....	4.36	3.32	3.11	2.97	2.94	2.84	2.75	2.64	2.53	2.36	2.18	2.06	1.87	1.66	1.50	1.40
Milligrams dissolved after 1 hour.....	42	55	86	249	265	348	408	...	547	538	401	366	272	219
Milligrams dissolved after 22 hours.....	102	133	164	267	342	459	536	634	646	733	788	779	710	528	374	300

of solid gelatin. Microscopic measurements of the influence of NaCl on the rate of swelling of individual grains of casein particles in $m/100$ HCl were made at 24°C., and the results plotted in Fig. 115. The ordinates are the average diameters of the particles after 1 and after 24 hours, respectively. The abscissæ are the concentrations of NaCl. The depressing effect is similar to that found in the case of the swelling of a jelly of gelatin. After 24 hours the particles had dissolved in the NaCl solutions of a concentration below $m/256$, but not in concentrations of NaCl higher than $m/256$.

These experiments show that it is necessary to overcome the cohesion between the casein molecules in acid before the granules can dissolve, and this is done by swelling. This conclusion was supported by measurements of the quantity of casein chloride dissolved at 20°C. at various pH of the solution. One gram of isoelectric powdered casein was put into 100 c.c. of solutions of HCl of different concentration and kept in these solutions in one case for 1 hour, in a second case for 22 hours. The mass was then poured into graduated cylinders and the undissolved part was allowed to settle to the bottom for 2 and for 6 hours, respectively, at 20°C. The supernatant liquid was removed and the sediment dried overnight in an oven at about 100°C. Table LX gives the result. The dry weight of 1 gm. of isoelectric casein was found to be 0.870 gm. and this weight minus the dry weight of the sediment was the amount dissolved. Table LX shows that the rate of solution increases with diminishing pH from 4.36 to 2.18 where the solubility of casein chloride is a maximum;

with a further decline in pH the solubility diminishes again. This is in agreement with the Donnan effect.

In a similar way the depressing effect of NaCl on the rate of solution of casein chloride was ascertained. Solutions of 12.5 c.c. of 0.1 N HCl in 100 c.c. and containing 1 gm. of powdered, originally isoelectric casein were prepared in 0, M/2,048, M/1,024, to M/4 NaCl. The pH of a solution of 1 gm. of casein in 100 c.c. containing 12.5 c.c. of 0.1 N HCl was 2.12 and this pH was the same in all solutions made up in NaCl. The solutions were kept at 20°C. for 16 hours and then allowed to settle for 24 hours at 20°C. in 100-c.c. graduated cylinders. The dry weight of the sediment was determined and this weight when deducted from the dry weight of 1 gm. of isoelectric casein, namely, 0.870 gm., was the amount that had gone into solution after a correction was made for the free NaCl held in a 2-c.c. solution which was arbitrarily assumed not to have been removed. Though this latter correction was somewhat arbitrary, it could have caused a noticeable error only when the concentration of the salt solution exceeded M/64. For the solutions of M/64 and below this error was negligible. Table LXI gives the number of milligrams of casein which had gone into solution.

TABLE LXI

	Concentration of NaCl					
	M/2,048	M/1,024	M/512	M/256	M/128	M/64
Milligrams dissolved.....	714	685	665	615	449	282

The main fact is that a slight increase in the concentration of NaCl causes a noticeable drop in the rate of solution. Thus, M/1,024 NaCl causes a noticeable diminution in the solubility of a 1 per cent solution of casein chloride of pH 2.12 at 20°C.

These observations, then, indicate that the solution of solid particles of casein chloride is brought about by the ultimate elements being forced apart mechanically through the process of swelling. The force acting in this swelling is the hydrostatic

pressure of the water which is forced into the interstices of the solid particles by the osmotic pressure of the solution in the interstices between the casein ions. As soon as the osmotic pressure in the particle exceeds the forces of cohesion between the casein ions of the particle, the casein ions constituting the particle are separated.

These experiments, then, show that in the case of the action of acid on peptization of casein particles the force causing the diminution of the particles is the hydrostatic pressure of the water driven into the particles osmotically. As soon as the osmotic forces are greater than the cohesion between the particles, peptization—or possibly true solution—occurs.

CHAPTER XX

SOME EXPERIMENTS ON SOLUTIONS OF PROTEIN IN ALCOHOL-WATER MIXTURES

It may be of interest to have some idea of the influence of the solvent on the properties of proteins, and as an illustration some experiments on alcoholic solutions of gelatin in mixtures of alcohol and water are here published. Since no measurements of potential differences—either membrane potentials or cataphoretic potentials—in alcoholic solutions are available, and since no pH measurements were possible, no theory of the behavior of the gelatin in alcohol can be given. The subject can at present be treated only empirically.

It was found that if a mixture of 1 c.c. of 1 per cent isoelectric gelatin plus 1 c.c. of H_2O , also of pH 4.7, was heated to about $35^{\circ}C.$, and 8 c.c. of absolute ethyl alcohol were added to the 2 c.c. of the isoelectric gelatin mixture in water (without salt), the gelatin was at once precipitated and a coagulation of the gelatin occurred, leaving the rest of the solution entirely clear. When, however, 1 c.c. of the 1 per cent isoelectric gelatin solution plus 1 c.c. of a salt solution, also of pH 4.7, were heated together and 8 c.c. of absolute alcohol were added, a more or less clear gelatin solution could be obtained when the proper salts were added. Such clearing effects were obtained with salts of the type of $CaCl_2$, $CeCl_3$, Na_2SO_4 , $Na_4Fe(CN)_6$, but not with salts like $NaCl$, KCl , $LiCl$, or $MgSO_4$. When too much salt was added the solution became opalescent, or opaque, or a precipitate occurred, according to the concentration of the salt. The 1 c.c. of salt solution added varied between concentrations of $M/32,768$ and 1 M . In the mixture the salt was diluted ten times, since 1 c.c. of isoelectric gelatin solution and 8 c.c. of ethyl alcohol were added to the 1-c.c. salt solution. The pH of the aqueous part of the solution was 4.7, that of the alcoholic solution could not be measured. Table LXII gives the results of the salt action.

We have seen in Chap. VI that isoelectric gelatin is sparingly soluble in water but becomes more soluble when a salt is added, and this solvent effect of the salt was the greater the greater the valency of one ion. In the case of alcohol-water mixtures, the solvent effect on gelatin at the isoelectric point seems to depend on the difference in the valency of the two oppositely charged ions of a salt, MgSO_4 or NaCl having no solvent effect, while Na_2SO_4 and MgCl_2 have a good solvent effect. In purely aqueous solution, MgSO_4 was as good a solvent of gelatin at the isoelectric point.

TABLE LXII. —CONCENTRATION OF SALT IN 100 C.C. OF THE SOLUTION IN WHICH THE PRECIPITATE WAS ENTIRELY OR ALMOST ENTIRELY DISSOLVED AT PH 4.7

MgCl_2	m/2,048 — m/512	Bluish, opalescent, not quite transparent
CaCl_2	m/1,024 — m/256	More transparent than MgCl_2
SrCl_2	m/1,024 — m/256	Like CaCl_2
BaCl_2	m/4,096 — m/512	Clearest of the four salts
LaCl_3	m/4,096 — m/32	Perfectly clear like water
Na_2SO_4	m/1,024 — m/256	Bluish and slightly opaque
$\text{Na}_4\text{Fe}(\text{CN})_6$	m/8,192 — m/128	Clear, at m/512 like water
LiCl	Flocculation in all concentrations	
NaCl		
KCl		
RbCl		
LiBr		
MgSO_4		

It may be that this clearing effect of the salt is due to an increase in the cataphoretic P.D. of the particles, but since no cataphoretic measurements are available, we cannot feel certain that this assumption is correct.

When the pH of the gelatin solution was either above 5.0 or below 4.4, and the anion of the acid or cation of the alkali added to isoelectric gelatin was monovalent, a considerable proportion of water could be replaced by absolute ethyl alcohol without causing a precipitate. The remarkable fact is that the more water is replaced by absolute ethyl alcohol the more salt is required for

precipitation of the gelatin, until finally a critical concentration is reached when quite abruptly the quantity of salt required for salting out is reduced to a mere trace. As soon as this critical concentration is reached, that ion of the salt which precipitates has the opposite sign of charge to that of the protein.

Ten per cent solutions of gelatin chloride of pH 3.0 and of Na gelatinate of pH 10.0 were prepared. Five cubic centimeters of such a solution were first warmed to liquefy the gelatin and then while still warm diluted with 45 c.c. of a mixture of alcohol and water, the relative quantity of alcohol and water in the 45 c.c. varying. Ten cubic centimeters of these 1 per cent solutions of gelatin chloride or Na gelatinate in water-alcohol were titrated with a solution of a neutral salt, $(\text{NH}_4)_2\text{SO}_4$, NaCl, and CaCl_2 , at 20°C., until precipitation occurred. It was noticeable that while it was not possible to precipitate the gelatin at all with $2\frac{1}{2}$ M CaCl_2 or 5 M NaCl, and only with comparatively high concentrations of $(\text{NH}_4)_2\text{SO}_4$, as long as the concentration of alcohol did not exceed a certain critical value, when this critical limit was exceeded traces of these salts sufficed for precipitation. This is illustrated in Tables LXIII and LXIV. When the solution contained no alcohol, *i.e.*, when 45 c.c. of H_2O were added to 5 c.c. of the 10 per cent solution of gelatin chloride of pH 3.0, 7.1 c.c. of 2 M $(\text{NH}_4)_2\text{SO}_4$ were required to cause precipitation (Table LXIII) in 10 c.c. of the 1 per cent gelatin chloride solution, and the quantity of $(\text{NH}_4)_2\text{SO}_4$ required increased at first the more H_2O was replaced by alcohol. When the 45 c.c. of liquid added to the 5 c.c. of 10 per cent gelatin solution consisted of 18.75 c.c. of water and 26.25 c.c. of ethyl alcohol, 17.8 c.c. of 2 M $(\text{NH}_4)_2\text{SO}_4$ were required to cause precipitation in 10 c.c. of the gelatin-alcohol-water mixture, but if now the proportion of alcohol to water was shifted only slightly in favor of alcohol, namely, 17.5 c.c. of water and 27.5 c.c. of alcohol, 0.04 c.c. instead of 17.8 c.c. of 2 M $(\text{NH}_4)_2\text{SO}_4$ sufficed for precipitation (Table LXIII).

In the case of NaCl the drop was still more striking. When the 45 c.c. added to the 5 c.c. of 10 per cent gelatin solution consisted of 8.75 c.c. of water and 36.25 c.c. of alcohol, it was impossible to cause precipitation in 10 c.c. of the gelatin chloride-alcohol-water mixture with any amount of 5 M NaCl.

When, however, the proportion of alcohol was only slightly increased, namely, 7.5 c.c. of water and 37.5 c.c. of alcohol, 0.2 c.c. of NaCl sufficed for precipitation. In the case of CaCl_2 the critical point was reached when the ratio was 5 c.c. of water and 40 c.c. of alcohol.

The existence of the critical point can be equally well demonstrated in the case of Na gelatinate, as is shown in Table LXIV.

What interests us is the following fact: The mechanism by which gelatin chloride of pH 3.0 and Na gelatinate of pH 10.0 are kept in solution is not altered as long as not too much of the water is replaced by alcohol, since in this case $(\text{NH}_4)_2\text{SO}_4$ is always a better precipitant than CaCl_2 for both gelatin chloride and Na gelatinate. When, however, the relative amount of alcohol exceeds a certain critical point, the mechanism by which the particles of gelatin are held in solution changes abruptly, as is indicated by two facts, namely first, that the concentration of the salt required for precipitation becomes suddenly very small, and second, that the efficient ion has now the opposite sign of charge to that of the colloidal particle. Thus, in the case of gelatin chloride (Table LXIII), the critical points for CaCl_2 and NaCl are close together, while the critical point for $(\text{NH}_4)_2\text{SO}_4$ is at a much lower concentration of alcohol. In this case the gelatin ion has a positive charge and the precipitating ion on the alcohol side of the critical point is the anion. In Table LXIV the critical points for $(\text{NH}_4)_2\text{SO}_4$ and for NaCl are close together, while the critical point for CaCl_2 lies at a much lower concentration of alcohol. In this case the colloidal ion is negatively charged and the precipitating ion of the salt is the cation.

It is not possible to say at present what happens at the critical alcohol concentration, whether, *e.g.*, the gelatin molecule undergoes a change whereby its solubility is diminished or lost.

When gelatin is dissolved in much alcohol and little water, so that the critical limit just discussed is exceeded, it differs in two respects from a gelatin solution in pure water: It has a comparatively low viscosity, and it no longer sets to a gel. It is also as a rule opalescent. The change in viscosity can be shown in the following way:

To 1 gm. of isoelectric gelatin enough HCl is added so that in a 1 per cent solution in water the pH would be about 3.0. This

gelatin is dissolved in mixtures of water and alcohol, heated rapidly to 45°C., and cooled rapidly to 15°C. The time of outflow through a viscometer is measured immediately at 15°C. As a control the time of outflow at 15°C. of identical water-alcohol mixtures, but containing no gelatin, is also measured. The ratio of the time of outflow of the gelatin-water-alcohol mixture to that of the water-alcohol mixture without gelatin, *i.e.*, the relative viscosity of the gelatin solution, is given in Table LXV. The upper horizontal row gives the relative amount of alcohol in per cent, the second row the appearance of the solution, the third the time of outflow of the gelatin solution in seconds, the fourth row the time of outflow of the alcohol-water mixture without gelatin, and the last row the relative viscosity of the gelatin solution. It is obvious that the viscosity drops sharply between 80 and 85 per cent of alcohol, and that at 85 per cent, where the solution is already opalescent, the relative viscosity is only 1.286 and only 1.1 for 87.5 per cent alcohol.

TABLE LXV.—INFLUENCE OF INCREASING QUANTITIES OF ALCOHOL ON THE VISCOSITY OF A 1 PER CENT SOLUTION OF GELATIN CHLORIDE OF ORIGINALLY PH 3.0

	Concentration of alcohol in per cent						
	0	40	70	80	85	87.5	90
Appearance of solution.....	clear				slightly opalescent	opalescent	very opalescent
Time of outflow of gelatin solution in seconds.....	207	266	506	362	229	185	163
Time of outflow of alcohol + water, without gelatin.....	80	233	225	194	178	168	160
Relative viscosity.....	2.590	2.860	2.250	1.860	1.286	1.100	1.020

In a second experiment the same solutions were prepared but the solutions were kept for 2 days in a thermostat at 9°C., the mixtures were then rapidly brought to 15°C., and the viscosities determined. The solutions containing 60 per cent of alcohol or less had set to a jelly; the solution containing 70 per cent was almost solid, but the solutions containing 80 per cent or more were all completely liquid. Their relative viscosity was measured (Table LXVI) and was found to be only slightly larger than at

the beginning, when the solution contained 85 per cent or more alcohol, while the viscosity had risen considerably when the solution contained less than 80 per cent alcohol.

The opalescence of the alcoholic solutions indicates the presence of aggregates of gelatin, but since the relative viscosity of these alcoholic solutions is low as compared with the viscosity of solutions of gelatin in pure water, and since the alcoholic solutions no longer set to a jelly, the micellæ in the aqueous solution and in the alcohol-water mixture, containing 80 per cent alcohol or more, must be different. The fact that the viscosity ratio is low in the opalescent gelatin-alcohol solutions (which no longer can set to a jelly) indicates that the micellæ in this solution occlude less water than the micellæ formed in the solutions of gelatin in water (or in water with not too much alcohol).

TABLE LXVI.—VISCOSITY AT 15°C. AFTER THE SOLUTION HAD BEEN KEPT AT 9°C. FOR 2 DAYS

	Concentration of alcohol		
	80 per cent	85 per cent	90 per cent
Appearance of solution.....	slightly opalescent	opalescent	very opalescent
Time of outflow of gelatin solution in seconds.....	521.0	247.0	180.0
Time of outflow of alcohol-water mixture without gelatin.....	194.0	178.0	160.0
Relative viscosity of gelatin solution...	2.685	1.390	1.125

This would suggest that the forces which hold gelatin in solution in pure water, or in water with little alcohol, are different from those which hold the gelatin in solution when the critical limit for alcohol has been exceeded. In aqueous solutions or in solutions with much water and little alcohol where setting of gelatin to a jelly is possible, the molecules or ions of jelly are distributed evenly in the solvent, probably on account of the strong forces of residual valency between solute and solvent. When, however, too much alcohol is added, *i.e.*, when the solution

is on the alcohol side of the critical point, the forces of attraction between gelatin and solvent are weakened to such an extent that the groups which were formerly attracted by the solvent are now more strongly attracted to each other than they are to the molecules of solvent. In the micellæ thus formed the protein ions or molecules are in much closer contact than they are in a jelly, and hence they occlude much less water than the micellæ formed in pure water or in mixtures of water with little alcohol. The latter micellæ increase the viscosity of the solution more than the micellæ formed in an excess of alcohol.

CHAPTER XXI

CONCLUDING REMARKS

Graham originally distinguished between crystalloidal and colloidal substances. This distinction lost its force when it was found that the same substance, *e.g.*, NaCl, forms true crystalloidal solutions in water but only suspensions in alcohol, and it became customary to distinguish between colloidal and crystalloidal states of matter. It was inferred that the same substance could be obtained either in the crystalloidal state or in the colloidal state, according to the nature of the solvent.

The results of the work discussed in this book show that the distinction between colloidal and crystalloidal states is also untenable and that we must distinguish between crystalloidal and colloidal behavior, since the same substance, *e.g.*, a protein, may show in regard to certain properties the general behavior of crystalloids and in respect to other properties the specific behavior attributed to colloids. Proteins behave like crystalloids in regard to the stoichiometrical character of their combination with acids and alkalies, as shown by the titration and combination curves. In this respect, proteins do not differ from the amino-acids of which they are composed and which are crystalloids. That this had been so long overlooked was due to the fact that the authors had failed to measure the hydrogen ion concentration of their protein solutions.

Proteins behave, furthermore, like amino-acids, that is, like crystalloids, in regard to their solubility. Like many ionizable crystalloids, proteins are more soluble when they are ionized than when they are not ionized, and for this reason their solubility is generally a minimum at the isoelectric point. A minimum of solubility at the isoelectric point exists also in the case of amino-acids (Michaelis). The fact that very often solid submicroscopic particles are formed in protein solutions does not contradict the crystalloidal nature of their solubility, since aggregate forma-

tion may occur in the aqueous solutions of true crystalloids, *e.g.*, cane sugar, if the proper concentration is reached. Moreover, solubility is a problem of the electronic configuration of the atoms and molecules and in the case of large molecules like proteins it is necessary to distinguish between the solubility of its different constituents which may vary considerably. Thus gelatin has apparently groups which have a high affinity for water, and also oily groups with a low affinity for water. These complications do not invalidate the fact that proteins generally behave like crystalloids, *i.e.*, amino-acids, in regard to solubility.

It is quite probable that proteins behave like crystalloids not only in regard to the stoichiometrical character of their chemical combination and their solubility, but also in regard to other properties. Reyher had shown that the viscosity of solutions of fatty acids increases with the ionization of the acids and this has been found true for amino-acids by Hedestrand. It is conceivable that this crystalloidal type of viscosity can be demonstrated also in the case of such proteins as crystalline egg albumin, the solutions of which are comparatively free from suspended solid particles.

Bottazzi has observed that the surface tension of protein solutions is a little lower at the isoelectric point than at any other pH. The writer has made similar experiments with egg albumin and has observed that while a 1 per cent solution of crystalline egg albumin had a surface tension of about 62 dynes at the isoelectric point, it had a surface tension of about 66 dynes at a pH of about 3.4, and at a pH of about 1.5 the surface tension dropped again to about 63 dynes. These variations are extremely small, and if they are a consequence of the ionization of the protein it might be possible that the same influence of the pH might be found in solutions of amino-acids or any other ionizable crystalloidal substances. It is also very likely that proteins show crystalloidal behavior in regard to the influence of electrolytes on cohesion, elasticity, etc., which depend on the electronic structure of matter.

It is, therefore, justifiable to consider proteins as crystalloids which show colloidal behavior only under a certain well-defined condition, namely, when there exists a block to the diffusion of the large protein ions but not to the diffusion of smaller crystalloi-

dal ions. Such a block may be produced by a membrane, such as collodion, impermeable to protein ions in solutions but permeable to small crystalloidal ions, or by the forces of cohesion between the molecules and ions of a protein gel which is easily permeable to crystalloidal ions. Such a condition leads to the establishment of a Donnan equilibrium in which the concentration of diffusible crystalloidal ions is greater in the protein solution or protein gel than in the outside aqueous solution free from protein. This difference in the molar concentration of crystalloidal ions inside and outside the protein solution or gel gives rise to the colloidal behavior of proteins, *i.e.*, to that behavior wherein they differ from crystalloids. This colloidal behavior includes the following properties: (1) membrane potentials, (2) osmotic pressure, (3) swelling, and (4) that form of viscosity which is due to the swelling of submicroscopic particles in the jelly. While these four properties are only a fraction of the many properties of protein solutions, they are very important. It is hardly necessary to point out that Donnan equilibria must play a rôle in the distribution of ions and of water in the body. Nevertheless, colloidal behavior is restricted to those conditions where the diffusion of one type of ions is prevented while no such block exists for the diffusion of other ions. The fact that the membranes which exist in the body or which can so easily be produced technically are liable to create such a block is the explanation for the universality of colloidal phenomena. It is, therefore, not correct to speak any longer of a chemistry of colloids or of a world of colloids. If we were in a position to produce membranes impermeable to Ca but permeable to Cl or Na ions, solutions containing CaCl_2 and NaCl would show colloidal behavior in respect to the influence of electrolytes on membrane potentials and on osmotic pressure when separated from pure water by such a membrane.

The establishment of a Donnan equilibrium is only possible when the protein is ionized and hence all the properties of proteins depending directly or indirectly on the Donnan equilibrium are a minimum at the isoelectric point. Crystalloidal properties of proteins which increase with ionization, such as solubility, have also a minimum at the isoelectric point. This has confused some authors who cannot see that all the properties of proteins, the value of which increases with increasing ionization, must be a

minimum at the isoelectric point because proteins are amphoteric electrolytes; but that ionization can give rise to a Donnan equilibrium and to colloidal behavior only under one condition, namely, when the diffusion of protein ions is prevented without prevention of diffusion of the other ions. Amino-acids will also show minimal values at their isoelectric point for all the properties which depend on ionization, yet they do not show colloidal behavior, for the simple reason that their ions can easily diffuse through those membranes through which protein ions cannot diffuse. If membranes are ever discovered which are impermeable to certain amino-acids but not to other crystalloidal ions, or if amino-acids are discovered which are capable of setting to a gel, it will then be possible to demonstrate colloidal behavior in amino-acids.

It has been customary to treat under the name of "colloid chemistry" not only properties due to the Donnan equilibrium, but also more general molecular properties, such as solubility, cohesion, adhesion, and surface tension, which have no direct connection with this theory. These molecular phenomena are part of electronic physics but are not specifically colloidal. They are influenced not only by the valency but also by the nature of the ion and differ in this respect from the purely colloidal phenomena due to the Donnan equilibrium, in which only the sign and valency of an ion are of importance.

The fact that the same substance in the same state can show crystalloidal as well as colloidal behavior will also clear up the confusion which exists in regard to the Hofmeister ion series. The Hofmeister series mean that both the valency and the chemical nature of the ions of a salt may influence the physical properties of a protein. This may be correct for strictly crystalloidal properties, such as the solubility or the cohesion of proteins; it is not correct, however, for the colloidal properties of proteins which depend on the Donnan equilibrium, for the simple reason that the Donnan equilibrium is an electrostatic equilibrium, which is influenced only by the number of charges, but not by the chemical nature of the ion.

The writer hopes that the methods, experimental results, and theoretical conclusions described in this book may be found of use not only in the study of the colloidal behavior of other

substances than proteins but also in physiology. Life phenomena cannot be dissociated from colloidal behavior, and the idea of an organism or of living matter consisting exclusively or chiefly of crystalloidal material or material with purely crystalloidal behavior is inconceivable. Organisms have been defined as chemical machines consisting essentially of collodial material capable of growing and automatically reproducing themselves.¹ If this be true, advance in general physiology will be chiefly a hit-or-miss game until science is in possession of a mathematical theory of the colloidal behavior of the substances of which living matter is composed. If Donnan's theory of membrane equilibria furnishes the mathematical and quantitative basis for a theory of colloidal behavior of the proteins, as the writer believes it does, it may be predicted that this theory will become one of the foundations on which modern physiology will have to rest.

¹ LOEB, J., "The Dynamics of Living Matter," New York, 1906.

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